PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 5/06, A61K 35/407 — A1

(11) International Publication Number: WO 96/37602

(43) International Publication Date: 28 November 1996 (28.11.96)

(21) International Application Number: PCT/US96/07590

(22) International Filing Date: 24 May 1996 (24.05.96)

(30) Priority Data:
08/451,915 26 May 1995 (26.05.95) US

(71) Applicant: DIACRIN, INC. [US/US]; Charlestown Navy Yard, Building 96, 13th Street, Charlestown, MA 02129 (US).

(72) Inventors: EDGE, Albert; 7 Ashton Place, Cambridge, MA 02138 (US). GUNSALUS, J., Ryan; 51 Revere Street, Boston, MA 02114 (US).

(74) Agents: SILVERI, Jean, M. et al.; Lahive & Cockfield, 60 State Street, Boston, MA 02109 (US).

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: PORCINE HEPATOCYTES FOR USE IN TREATMENT OF DISORDERS CHARACTERIZED BY INSUFFICIENT LIVER FUNCTION

(57) Abstract

Isolated porcine hepatocytes, isolated populations of such hepatocytes and methods for using the hepatocytes to treat subjects with disorders characterized by insufficient liver function are described. The porcine hepatocytes can be either hepatocytes isolated from adult, immature, or embryonic swine. The porcine hepatocytes can be modified to be suitable for transplantation into a xenogenic subject, for example, by altering an antigen (e.g., an MHC class I antigen) on the cell surface which is capable of stimulating an immune response against the cell in the subject (e.g, by contact with an anti-MHC class I antibody, or a fragment or derivative thereof). The isolated porcine hepatocytes of the invention can be used to treat disorders characterized by insufficient liver function by administering the hepatocytes to a subject having such a disorder.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

PORCINE HEPATOCYTES FOR USE IN TREATMENT OF DISORDERS CHARACTERIZED BY INSUFFICIENT LIVER FUNCTION

Background of the Invention

Vulnerable to a wide variety of metabolic, circulatory, toxic, microbial, and neoplastic insults, the liver is one of the most frequently injured organs in the body. In some instances, the disease is primarily localized in liver cells. For example, primary liver diseases include hereditary disorders such as Gilbert's Syndrome, Crigler-Najjar Syndrome (either Type I or Type II), Dubin Johnson Syndrome, familial hypercholesterolemia, ornithine transcarbamoylase (OTC) deficiency, hereditary emphysema and hemophilia; viral infections such as hepatitis A, B, and non-A, non-B hepatitis; and hepatic malignancies such as hepatocellular carcinoma. Robbins, S.L. et al. (1984) Pathologic Basis of Disease (W.B. Saunders Company, Philadelphia) pp. 884-942. More often, the hepatic involvement is secondary, often to some of the most common diseases of man, such as cardiac decompensation, disseminated cancer, alcoholism, and extrahepatic infections. Robbins, S.L. et al. (1984) Pathologic Basis of Disease (W.B. Saunders Company, Philadelphia) pp. 884-942.

One of the more devastating of the above-listed liver diseases is familial hypercholesterolemia (FH). FH is a consequence of a mutation in the low density lipoprotein (LDL) receptor locus. Brown, M.S. et al. (1986) *Science* 232:34-47. The LDL receptor is a specific cell membrane receptor involved in the transport and metabolism of cholesterol. FH heterozygotes with one mutant allele (representing about one in 500 individuals) have, from birth, a two- to threefold elevation of plasma cholesterol leading to premature atherosclerosis and coronary heart disease in adult life. Grundy, S. M. et al. *JAMA* 269:3015-3023. Myocardial infarctions begin to occur in heterozygous men in the third decade and peak in the fourth and fifth decades. By the age of sixty, approximately eighty-five percent of men have experienced a myocardial infarction. Women also experience an increased incidence of myocardial infarction, but the mean age of onset is ten years later.

FH homozygotes have two mutant alleles at the LDL receptor locus and are much more severely affected. These FH homozygotes have five- to sixfold elevations in plasma cholesterol levels. These individuals develop coronary, cerebral, and peripheral vascular atherosclerosis at an early age. FH homozygotes have marked elevations of LDL in the plasma from birth. Total cholesterol levels in homozygous FH are typically greater than 500 mg/dl (normal is 200 mg/dl), and the patients frequently have decreased high density lipoprotein levels. FH homozygotes typically succumb to complications from coronary heart disease prior to age 20, with males developing the disease earlier than females. Robbins, S.L. et al. (1984) Pathologic Basis of Disease (W.B. Saunders Company, Philadelphia) pp.139-140.

While drug therapy is available for FH heterozygotes, normal levels of plasma cholesterol are difficult to achieve. In the case of the FH homozygotes, the condition cannot be treated by conventional drug therapy, and the therapeutic recourses are limited to chronic plasmapheresis or orthotopic liver transplantation.

Whole liver transplantation, which is the current therapy for a variety of liver diseases, has been employed to successfully reconstitute LDL receptors in individuals with FH, thereby lowering serum cholesterol to normal levels. Whole liver transplantation, however, is limited by the scarcity of suitable donor organs. Li, Q. et al. (1993) *Human Gene Therapy* 4:403-409; Kay, M.A. et al. (1992) *Proc. Natl. Acad. Sci.* 89:89-93. In addition to the difficulty in obtaining donor organs, the expense of liver transplantation, estimated at approximately $200,000 to $300,000 per procedure, prohibits its widespread application. Another unsolved problem is graft rejection. Foreign livers and liver cells are poorly tolerated by the recipient and are rapidly destroyed by the immune system in the absence of immunosuppressive drugs. Li, Q. et al. (1993) *Human Gene Therapy* 4:403-409; Bumgardner, G.L. et al. (1992) *Transplantation* 53:857-862. While immunosuppressive drugs may be used to prevent rejection, they also block desirable immune responses such as those against bacterial and viral infections, thereby placing the recipient at risk of infection. There is a clear need, therefore, to address the limitations of the current liver transplantation therapy as treatment for the vast array of liver disorders.

Summary of the Invention

To overcome the current limitations of whole liver transplantation to treat liver disorders, the present invention provides hepatocytes, compositions including the hepatocytes, and methods for treating disorders characterized by insufficient liver function by administering the hepatocytes to subjects with such disorders. The hepatocytes of the present invention offer several advantages over whole liver transplantation to treat liver disorders. For example, the hepatocytes of the present invention are isolated from pigs, which provide a convenient, relatively inexpensive, and abundant source of hepatocytes. Moreover, in some instances, the hepatocytes of the present invention are modified such that rejection of the hepatocytes upon introduction into a xenogeneic recipient is inhibited, thereby eliminating the requirement for generalized suppression of the immune system.

Accordingly, the present invention pertains to an isolated porcine hepatocyte or an isolated population of porcine hepatocytes suitable for transplantation into a xenogeneic subject, particularly a human subject. In a preferred embodiment, the xenogeneic subject has a disorder characterized by insufficient liver function. Examples of such disorders include hereditary disorders such as Gilbert's Syndrome, Crigler-Najjar Syndrome (either Type I or Type II), Dubin Johnson Syndrome, familial hypercholesterolemia, ornithine transcarbamoylase (OTC) deficiency, hereditary emphysema, and hemophilia; viral hepatitis, such as hepatitis A, B, and non-A, non-B hepatitis, hepatocellular carcinoma, acute liver

failure, and chronic liver failure. The porcine hepatocyte(s), in unmodified form, has at least one antigen on the cell surface which is capable of stimulating an immune response against the cell in a xenogeneic subject, for example, a human. The antigen on the surface of the porcine hepatocyte is altered to inhibit rejection of the cell when introduced into a xenogeneic subject. In one embodiment, the cell surface antigen which is altered is an MHC class I antigen. This MHC class I antigen can be contacted, prior to transplantation into a xenogeneic subject, with at least one anti-MHC class I antibody, or a fragment or derivative thereof, which binds to the MHC class I antigen on the cell surface but does not activate complement or induce lysis of the cell. One example of an anti-MHC class I antibody is an anti-MHC class I $F(ab')_2$ fragment, such as an anti-MHC class I $F(ab')_2$ fragment of a monoclonal antibody PT85. The present invention also pertains to compositions which include porcine hepatocytes and antibodies, antibody fragments, or derivatives, which bind an antigen on the surface of the porcine hepatocytes. These compositions can be inserted into a delivery device, e.g., a syringe, e.g., a syringe pump, which facilitates the introduction of the cells into a subject. In addition, the porcine hepatocytes of the invention can be grown as a cell culture in a medium suitable to support the growth of the cells.

Porcine hepatocytes obtained from both embryonic (i.e., fetal), newborn, and adult pigs are suitable for transplantation into a xenogeneic subject. Typically, embryonic porcine hepatocytes are isolated during selected stages of gestational development. For example, hepatocytes can be isolated from an embryonic pig at a stage of embryonic development when the cells can be recognized as hepatocytes. In one embodiment, the hepatocytes are isolated between about day twenty (20) to about day twenty-five (25) of gestation and birth of the pig. In other preferred embodiments, the hepatocytes are isolated between about day thirty (30) to about day thirty-five (35) of gestation and birth of the pig, more preferably between about day twenty-five (25) and about day ninety (90) of gestation, still more preferably between about day thirty (30) and about day eighty (80) of gestation, yet more preferably between about day thirty-five (35) and about day seventy (70) of gestation, still further preferably between about day thirty-five (35) and about day fifty (50) to about day sixty (60) of gestation, and most preferably between about day thirty-five (35) and about day forty (40) of gestation.

The invention further pertains to an isolated porcine hepatocyte or an isolated population of hepatocytes isolated from a pig which is essentially free from organisms which are capable of transmitting infection or disease to a xenogeneic recipient, e.g., a human, of the cells. Categories of pathogens from which the pig are free can include parasites, bacteria, mycoplasma, and viruses. In one embodiment, the pig from which the hepatocytes are isolated is free of the following organisms: Toxoplasma, eperythrozoon, brucella, listeria, mycobacterium TB, leptospirillum, haemophilus suis, M. Hyopneumonia, porcine respiratory reproductive syndrome, rabies, pseudorabies, parvovirus, encephalomyocarditus virus, swine vesicular disease, techen (Porcine polio virus), hemagglutinating encephalomyocarditus,

suipoxvirus, swine influenza type A, adenovirus, transmissible gastroenteritis virus, bovine viral diarrhea, and vesicular stomatitis virus. The cells obtained from pathogen-free pigs can be modified as described herein to inhibit rejection of the cell upon introduction into a xenogeneic subject. Preferred hepatocyte ages are described herein. The present invention also pertains to compositions which include porcine hepatocytes obtained from pathogen-free pigs and antibodies, antibody fragments, or derivatives, which bind an antigen on the surface of the porcine hepatocytes. These compositions can also be inserted into a delivery device, e.g., a syringe, e.g., a syringe pump, which facilitates the introduction of the cells into a subject.

Another aspect of the invention pertains to methods for treating disorders characterized by insufficient liver function, e.g., hereditary disorders such as Gilbert's Syndrome, Crigler-Najjar Syndrome (either Type I or Type II), Dubin Johnson Syndrome, familial hypercholesterolemia, OTC deficiency, hereditary emphysema and hemophilia; viral infections such as hepatitis A, B, and non-A, non-B hepatitis; hepatic malignancies such as hepatocellular carcinoma, acute liver failure, and chronic liver failure, in a subject, particularly a human subject. These methods include administering to a subject having such a disorder, an isolated population of porcine hepatocytes. In one embodiment, the porcine hepatocytes which can be administered to a subject having a liver disorder are porcine hepatocytes which, in unmodified form, have at least one antigen on the cell surface which is capable of stimulating an immune response against the cell in a xenogeneic subject, for example, a human. The antigen on the surface of the porcine hepatocyte is altered to inhibit rejection of the cell when introduced into a xenogeneic subject. Examples of hepatocyte cell surface antigens and methods of altering such antigens are described herein. Preferred hepatocyte ages are also described herein. In another embodiment, the porcine hepatocytes which can be administered to a subject having a disorder characterized by insufficient liver function are porcine hepatocytes which are obtained from a pig which is essentially free from organisms which are capable of transmitting infection or disease to a xenogeneic recipient, e.g., a human, of the cells. Pathogen-free pigs are described in detail herein. Transplantation of the porcine hepatocytes can be accompanied by administration of an immunosuppressive agent, e.g., cyclosporine A, FK506, RS-61443, or a T cell antibody, to the subject.

Brief Description of the Drawings

Figure 1 is a Western blot showing porcine albumin production in a Watanabe heritable hyperlipidemic (WHHL) rabbit transplanted with porcine hepatocytes and treated with cyclosporin.

Figures 2A-2F show localization of porcine albumin in liver sections of the WHHL rabbit transplanted with porcine hepatocytes and treated with cyclosporin.

Figure 3 is a graphic representation of total serum cholesterol levels in WHHL rabbits transplanted with porcine hepatocytes and treated with cyclosporin.

Figure 4 is a graphic representation of the long term-effect of porcine hepatocyte transplantation in combination with cyclosporin treatment on total serum cholesterol in the WHHL rabbit.

Figure 5 is a graphic representation of the effect of cyclosporin therapy on hepatic graft survival as detected by decreases in total serum cholesterol in the WHHL rabbit.

Figure 6 is a graphic representation of total serum cholesterol levels in WHHL rabbits after a second porcine hepatocyte transplantation and cyclosporin treatment.

Figure 7 is a graphic representation of the clearance of ^{125}I-human low density lipoprotein (hLDL) from serum by a WHHL rabbit and a New Zealand White rabbit, neither of which was transplanted with porcine hepatocytes, and a WHHL rabbit transplanted with porcine hepatocytes and treated with cyclosporin.

Figure 8 is a graphic representation of the lipoprotein profiles in the WHHL and New Zealand White rabbit transplanted with porcine hepatocytes and treated with cyclosporin as determined by FPLC.

Figure 9 is a graphic representation of total serum cholesterol levels in WHHL rabbits transplanted with modified (i.e., masked) porcine hepatocytes.

Figure 10 is a graphic representation of the effect of transplantation of masked porcine hepatocytes and administration of a subtherapeutic regimen of cyclosporin A on total serum cholesterol levels in WHHL rabbits.

Figure 11 is a graphic representation of the clearance of ^{125}I-hLDL from serum by a WHHL rabbit and a New Zealand White rabbit, neither of which was transplanted with porcine hepatocytes, and a WHHL rabbit transplanted with masked porcine hepatocytes.

Figure 12 is a graphic representation of the lipoprotein profiles in the transplanted WHHL and New Zealand White rabbit transplanted with masked porcine hepatocytes as determined by FPLC.

Figures 13A-13D show localization of porcine hepatocytes in liver sections from a WHHL rabbit transplanted with masked porcine hepatocytes by *in situ* hybridization using a porcine repeat element.

Figure 14 is a blot illustrating the presence of porcine repeat element in DNA isolated from the liver of a WHHL rabbit transplanted with masked porcine hepatocytes nine months prior to sacrifice and a control WHHL rabbit.

Detailed Description of the Invention

I. ISOLATED CELLS AND CELL POPULATIONS OF THE INVENTION

A. Porcine Hepatocytes Suitable for Administration to Xenogeneic Subjects

This invention features an isolated porcine hepatocyte and an isolated population of porcine hepatocytes which are suitable for administration to a xenogeneic subject. These

cells can be used to treat disorders which are characterized by insufficient liver function. As used herein, the term "isolated" refers to a cell which has been separated from its natural environment. This term includes gross physical separation from its natural environment, e.g., removal from the donor animal, e.g., a pig, and alteration of the cell's relationship with the neighboring cells with which it is in direct contact by, for example, dissociation. The term "isolated" when used herein to refer to cell does not refer to a cell which is in a tissue section, is cultured as part of a tissue section, or is transplanted in the form of a tissue section. As used herein, the term "porcine" is used interchangeably with the terms "pig" and "swine" and refers to mammals in the family Suidae. Such mammals include wholly or partially inbred swine, e.g., miniature swine, and transgenic swine.

Hepatocytes are some of the most versatile cells in the body. Hepatocytes have both endocrine and exocrine functions, and synthesize and accumulate certain substance, detoxify others, and secrete others to perform enzymatic, transport, or hormonal activities. The main activities of liver cells include bile secretion, regulation of carbohydrate, lipid, and protein metabolism, storage of substances important in metabolism, degradation and secretion of hormones, and transformation and excretion of drugs and toxins. The term "hepatocyte" as used herein refers to a liver parenchymal cell.

Hepatocytes of the invention are obtained or isolated from the liver of a donor swine such as, for example, a swine which is essentially pathogen-free as described herein. Hepatocytes can be obtained or isolated from both adult, newborn (immature), and embryonic (i.e., fetal) swine. Embryonic hepatocytes are obtained from the liver of an embryonic donor swine and preferably at a selected gestational age. The selected gestational ages (the total gestation time for pig is approximately 115 days) for obtaining fetal or embryonic hepatocytes are determined based on the following criteria: the ability of the embryonic porcine liver structure to be identified; the viability of the cells upon isolation from the donor pig, the ability of the cells to proliferate in culture; the stage of development of the fetal liver is such that at least about 50% or more of the cells isolated therefrom are hepatocytes. The preferred gestational age of embryonic swine from which to obtain hepatocytes suitable for introduction into xenogeneic subjects, particularly humans, is between about day twenty (20) to about day twenty-five (25) of gestation and birth of the pig. Hepatocytes are preferably isolated between about day thirty (30) to about day thirty-five (35) of gestation and birth of the pig, more preferably between about day twenty-five (25) and about day ninety (90) of gestation, still more preferably between about day thirty (30) and about day eighty (80) of gestation, yet more preferably between about day thirty-five (35) and about day seventy (70) of gestation, still further preferably between about day thirty-five (35) and about day fifty (50) to about day sixty (60) of gestation, and most preferably between about day thirty-five (35) and about day forty (40) of gestation.

When isolated from the a donor swine, the hepatocytes of the invention are capable of, among other functions, proliferating, secreting plasma proteins, such as albumin,

expressing low density lipoprotein receptors and thus, binding low density lipoproteins, maintaining differentiated functions *in vivo,* and adhering to substrates, such as culture dishes. When isolated from a donor swine, the hepatocytes of the present invention also have an epithelial morphology and are binucleate.

The present invention also features a population of porcine hepatocytes. As used herein the term "population" refers to a group of two or more cells. The cells of the invention can be maintained as a functionally viable cell culture. The characteristics of the cells when grown as cell cultures are described herein in detail. Media which can be used to support the growth of porcine hepatocytes include mammalian cell culture media, such as those produced by Gibco BRL (Gaithersburg, MD). *See* 1994 Gibco BRL Catalogue & Reference Guide. The medium can be serum-free but is preferably supplemented with animal serum such as fetal calf serum. A preferred medium is DMEM/Weymouths supplemented with fetal calf serum. When isolated from a donor pig and/or when maintained in culture, preferably at least about 20%, more preferably at least about 30%, yet more preferably at least about 40%, still more preferably at least about 50%, and most preferably at least about 60% or more of the hepatocytes express at least one liver-specific protein, e.g., albumin, LDL receptor.

The hepatocytes of the invention can be further included in compositions. For example, such compositions can include antibodies, antibody fragments, or derivatives, which bind to at least one antigen on the hepatocyte surface which is capable of stimulating an immune response against the hepatocyte in a xenogeneic subject. Hepatocyte surface antigens are described herein in detail. In one embodiment, the compositions can also include a pharmaceutically acceptable carrier or diluent as described herein.

Hepatocytes of the invention can also be "modified to express a gene product". As used herein, the term "modified to express a gene product" is intended to mean that the cell is treated in a manner that results in the production of a gene product by the cell. Preferably, the cell does not express the gene product prior to modification. Alternatively, modification of the cell may result in an increased production of a gene product already expressed by the cell or result in production of a gene product (e.g., an antisense RNA molecule) which decreases production of another, undesirable gene product normally expressed by the cell.

In a preferred embodiment, a cell is modified to express a gene product by introducing genetic material, such as a nucleic acid molecule (e.g., RNA or, more preferably, DNA) into the cell. The nucleic acid molecule introduced into the cell encodes a gene product to be expressed by the cell. The term "gene product" as used herein is intended to include proteins, peptides and functional RNA molecules. Generally, the gene product encoded by the nucleic acid molecule is the desired gene product to be supplied to a subject. Alternatively, the encoded gene product is one which induces the expression of the desired gene product by the cell (e.g., the introduced genetic material encodes a transcription factor which induces the transcription of the gene product to be supplied to the subject). Examples of gene products that can be delivered to a subject via a modified hepatocyte include low

density lipoprotein receptors, blood clotting Factor VIII and Factor IX, UDP glucuronyl transferase, ornithine transcarbamoylase, and cytochrome p450 enzymes.

A nucleic acid molecule introduced into a cell is in a form suitable for expression in the cell of the gene product encoded by the nucleic acid. Accordingly, the nucleic acid molecule includes coding and regulatory sequences required for transcription of a gene (or portion thereof) and, when the gene product is a protein or peptide, translation of the gene product encoded by the gene. Regulatory sequences which can be included in the nucleic acid molecule include promoters, enhancers and polyadenylation signals, as well as sequences necessary for transport of an encoded protein or peptide, for example N-terminal signal sequences for transport of proteins or peptides to the surface of the cell or for secretion.

Nucleotide sequences which regulate expression of a gene product (e.g., promoter and enhancer sequences) are selected based upon the type of cell in which the gene product is to be expressed and the desired level of expression of the gene product. For example, a promoter known to confer cell-type specific expression of a gene linked to the promoter can be used. A promoter specific for myoblast gene expression can be linked to a gene of interest to confer muscle-specific expression of that gene product. Muscle-specific regulatory elements which are known in the art include upstream regions from the dystrophin gene (Klamut et al., (1989) *Mol. Cell. Biol.* 9:2396), the creatine kinase gene (Buskin and Hauschka, (1989) *Mol. Cell Biol.* 9:2627) and the troponin gene (Mar and Ordahl, (1988) *Proc. Natl. Acad. Sci. USA.* 85:6404). Regulatory elements specific for other cell types are known in the art (e.g., the albumin enhancer for liver-specific expression; insulin regulatory elements for pancreatic islet cell-specific expression; various neural cell-specific regulatory elements, including neural dystrophin, neural enolase and A4 amyloid promoters). Alternatively, a regulatory element which can direct constitutive expression of a gene in a variety of different cell types, such as a viral regulatory element, can be used. Examples of viral promoters commonly used to drive gene expression include those derived from polyoma virus, Adenovirus 2, cytomegalovirus and Simian Virus 40, and retroviral LTRs. Alternatively, a regulatory element which provides inducible expression of a gene linked thereto can be used. The use of an inducible regulatory element (e.g., an inducible promoter) allows for modulation of the production of the gene product in the cell. Examples of potentially useful inducible regulatory systems for use in eukaryotic cells include hormone-regulated elements (e.g., see Mader, S. and White, J.H. (1993) *Proc. Natl. Acad. Sci. USA* 90:5603-5607), synthetic ligand-regulated elements (see, e.g. Spencer, D.M. et al. (1993) *Science* 262:1019-1024) and ionizing radiation-regulated elements (e.g., see Manome, Y. et al. (1993) *Biochemistry* 32:10607-10613; Datta, R. et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:10149-10153). Additional tissue-specific or inducible regulatory systems which may be developed can also be used in accordance with the invention.

There are a number of techniques known in the art for introducing genetic material into a cell that can be applied to modify a cell of the invention. In one embodiment, the

nucleic acid is in the form of a naked nucleic acid molecule. In this situation, the nucleic acid molecule introduced into a cell to be modified consists only of the nucleic acid encoding the gene product and the necessary regulatory elements. Alternatively, the nucleic acid encoding the gene product (including the necessary regulatory elements) is contained within a plasmid vector. Examples of plasmid expression vectors include CDM8 (Seed, B., *Nature* 329:840 (1987)) and pMT2PC (Kaufman, et al., *EMBO J.* 6:187-195 (1987)). In another embodiment, the nucleic acid molecule to be introduced into a cell is contained within a viral vector. In this situation, the nucleic acid encoding the gene product is inserted into the viral genome (or a partial viral genome). The regulatory elements directing the expression of the gene product can be included with the nucleic acid inserted into the viral genome (i.e., linked to the gene inserted into the viral genome) or can be provided by the viral genome itself.

Naked DNA can be introduced into cells by forming a precipitate containing the DNA and calcium phosphate. Alternatively, naked DNA can also be introduced into cells by forming a mixture of the DNA and DEAE-dextran and incubating the mixture with the cells. or by incubating the cells and the DNA together in an appropriate buffer and subjecting the cells to a high-voltage electric pulse (i.e., by electroporation). A further method for introducing naked DNA cells is by mixing the DNA with a liposome suspension containing cationic lipids. The DNA/liposome complex is then incubated with cells. Naked DNA can also be directly injected into cells by, for example, microinjection. For cells in culture, DNA can be introduced by microinjection. For cells *in vivo*, DNA can be introduced through the use of a gene gun.

Alternatively, naked DNA can also be introduced into cells by complexing the DNA to a cation, such as polylysine, which is coupled to a ligand for a cell-surface receptor (see for example Wu, G. and Wu, C.H. (1988) *J. Biol. Chem.* 263:14621; Wilson et al. (1992) *J. Biol. Chem.* 267:963-967; and U.S. Patent No. 5,166,320). Binding of the DNA-ligand complex to the receptor facilitates uptake of the DNA by receptor-mediated endocytosis. An alternative method for generating a cell that is modified to express a gene product involving introducing naked DNA into cells is to create a transgenic animal which contains cells modified to express the gene product of interest.

Use of viral vectors containing nucleic acid, e.g., a cDNA encoding a gene product, is a preferred approach for introducing nucleic acid into a cell. Infection of cells with a viral vector has the advantage that a large proportion of cells receive the nucleic acid, which can obviate the need for selection of cells which have received the nucleic acid. Additionally, molecules encoded within the viral vector, e.g., by a cDNA contained in the viral vector, are expressed efficiently in cells which have taken up viral vector nucleic acid and viral vector systems can be used either *in vitro* or *in vivo*.

Defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) *Blood* 76:271). A recombinant retrovirus can be constructed having a nucleic acid encoding a gene product of interest inserted into the

retroviral genome. Additionally, portions of the retroviral genome can be removed to render the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques.

The genome of an adenovirus can be manipulated such that it encodes and expresses a gene product of interest but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See for example Berkner et al. (1988) *BioTechniques* 6:616; Rosenfeld et al. (1991) *Science* 252:431-434; and Rosenfeld et al. (1992) *Cell* 68:143-155. Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Recombinant adenoviruses are advantageous in that they do not require dividing cells to be effective gene delivery vehicles and can be used to infect a wide variety of cell types, including airway epithelium (Rosenfeld et al. (1992) cited *supra*), endothelial cells (Lemarchand et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:6482-6486), hepatocytes (Herz and Gerard (1993) *Proc. Natl. Acad. Sci. USA* 90:2812-2816) and muscle cells (Quantin et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:2581-2584). Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (e.g., retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner et al. cited *supra*; Haj-Ahmand and Graham (1986) *J. Virol.* 57:267). Most replication-defective adenoviral vectors currently in use are deleted for all or parts of the viral E1 and E3 genes but retain as much as 80 % of the adenoviral genetic material.

Adeno-associated virus (AAV) is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review see Muzyczka et al. *Curr. Topics in Micro. and Immunol.* (1992) 158:97-129). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (see for example Flotte et al. (1992) *Am. J. Respir. Cell. Mol. Biol.* 7:349-356; Samulski et al. (1989) *J. Virol.* 63:3822-3828; and McLaughlin et al. (1989) *J. Virol.* 62:1963-1973). Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin et al. (1985) *Mol. Cell. Biol.* 5:3251-3260 can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat et al. (1984) *Proc. Natl. Acad. Sci. USA* 81:6466-6470; Tratschin et al. (1985) *Mol. Cell. Biol.* 4:2072-2081; Wondisford et al. (1988) *Mol. Endocrinol.* 2:32-39; Tratschin et al. (1984) *J. Virol.* 51:611-619; and Flotte et al. (1993) *J. Biol. Chem.* 268:3781-3790).

When the method used to introduce nucleic acid into a population of cells results in modification of a large proportion of the cells and efficient expression of the gene product by the cells (e.g., as is often the case when using a viral expression vector), the modified population of cells may be used without further isolation or subcloning of individual cells within the population. That is, there may be sufficient production of the gene product by the population of cells such that no further cell isolation is needed. Alternatively, it may be desirable to grow a homogenous population of identically modified cells from a single modified cell to isolate cells which efficiently express the gene product. Such a population of uniform cells can be prepared by isolating a single modified cell by limiting dilution cloning followed by expanding the single cell in culture into a clonal population of cells by standard techniques.

Alternative to introducing a nucleic acid molecule into a cell to modify the cell to express a gene product, a cell can be modified by inducing or increasing the level of expression of the gene product by a cell. For example, a cell may be capable of expressing a particular gene product but fails to do so without additional treatment of the cell. Similarly, the cell may express insufficient amounts of the gene product for the desired purpose. Thus, an agent which stimulates expression of a gene product can be used to induce or increase expression of a gene product by the cell. For example, cells can be contacted with an agent *in vitro* in a culture medium. The agent which stimulates expression of a gene product may function, for instance, by increasing transcription of the gene encoding the product, by increasing the rate of translation or stability (e.g., a post transcriptional modification such as a poly A tail) of an mRNA encoding the product or by increasing stability, transport or localization of the gene product. Examples of agents which can be used to induce expression of a gene product include cytokines and growth factors.

Another type of agent which can be used to induce or increase expression of a gene product by a cell is a transcription factor which upregulates transcription of the gene encoding the product. A transcription factor which upregulates the expression of a gene encoding a gene product of interest can be provided to a cell, for example, by introducing into the cell a nucleic acid molecule encoding the transcription factor. Thus, this approach represents an alternative type of nucleic acid molecule which can be introduced into the cell (for example by one of the previously discussed methods). In this case, the introduced nucleic acid does not directly encode the gene product of interest but rather causes production of the gene product by the cell indirectly by inducing expression of the gene product.

B. Modified Porcine Hepatocytes and Isolated Populations of Modified Porcine Hepatocytes

A further aspect of the invention is a porcine hepatocyte (and an isolated population of porcine hepatocytes) which, in unmodified form, has at least one antigen on the cell surface which is capable of stimulating an immune response against the cell in a xenogeneic

subject. To inhibit rejection of the cell when introduced into the xenogeneic subject, the antigen on the cell surface is altered prior to transplantation. In an unaltered state, the antigen on the cell surface stimulates an immune response against the cell when the cell is administered to a subject. By altering the antigen, the normal immunological recognition of the porcine hepatocyte by the immune system cells of the recipient is disrupted and additionally, "abnormal" immunological recognition of this altered form of the antigen can lead to porcine hepatocyte-specific long term unresponsiveness in the recipient. It is likely that alteration of an antigen on the porcine hepatocyte prior to introducing the cell into a subject interferes with the initial phase of recognition of the porcine hepatocyte by the cells of the host's immune system subsequent to administration of the cell. Furthermore, alteration of the antigen can induce immunological nonresponsiveness or tolerance, thereby preventing the induction of the effector phases of an immune response (e.g., cytotoxic T cell generation, antibody production etc.) which are ultimately responsible for rejection of foreign cells in a normal immune response. As used herein, the term "altered" encompasses changes that are made to at least one porcine hepatocyte antigen(s) which reduce the immunogenicity of the antigen to thereby interfere with immunological recognition of the antigen(s) by the recipient's immune system. An example of an alteration of a porcine hepatocyte antigen is binding of a second molecule to the antigen. The second molecule can decrease or prevent recognition of the antigen as a foreign antigen by the recipient subject's immune system.

Antigens to be altered according to the current invention include antigens on a hepatocyte which can interact with an immune cell in a xenogeneic (or allogeneic) recipient subject and thereby stimulate a specific immune response against the porcine hepatocyte in the recipient. The interaction between the antigen and the immune cell can be an indirect interaction (e.g., mediated by soluble factors which induce a response in the immune cell, e.g., humoral mediated) or, preferably, is a direct interaction between the antigen and a molecule present on the surface of the immune cell (i.e., cell-cell mediated). As used herein, the term "immune cell" is intended to include a cell which plays a role in specific immunity (e.g., is involved in an immune response) or plays a role in natural immunity. Examples of immune cells include all distinct classes of lymphocytes (T lymphocytes, such as helper T cells and cytotoxic T cells, B lymphocytes, and natural killer cells), monocytes, macrophages, other antigen presenting cells, dendritic cells, and leukocytes (e.g., neutrophils, eosinophils, and basophils). In a preferred embodiment, the antigen is one which interacts with a T lymphocyte in the recipient (e.g., the antigen normally binds to a receptor on the surface of a T lymphocyte).

In one embodiment, the antigen on the porcine hepatocyte to be altered is an MHC class I antigen. Alternatively, an adhesion molecule on the cell surface, such as ICAM-1, can be altered. An antigen which stimulates a cellular immune response against the cell, such as an MHC class I antigen, can be altered prior to transplantation by contacting the cell with a molecule which binds to the antigen. A preferred molecule for binding to the antigen is an

antibody, or fragment thereof (e.g., an MHC class I antibody, or fragment thereof). A preferred antibody fragment is an $F(ab')_2$ fragment. Polyclonal or, more preferably, monoclonal antibodies can be used. Other molecules which can be used to alter an antigen (e.g., an MHC class I antigen) include peptides and small organic molecules which bind to the antigen. Furthermore, two or more different epitopes on the same or different antigens on the cell surface can be altered. A particularly preferred monoclonal antibody for alteration of MHC class I antigens on porcine hepatocytes is PT85 (commercially available from Veterinary Medicine Research Development, Pullman WA). PT85 can be used alone to alter MHC class I antigens or, if each antibody is specific for a different epitope, PT85 can be used in combination with another antibody known to bind MHC class I antigens to alter the antigens on the cell surface. Suitable methods for altering a surface antigen on a cell for transplantation are described in greater detail in Faustman and Coe (1991) *Science* 252:1700-1702 and PCT publication WO 92/04033. Methods for altering multiple epitopes on a surface antigen on a cell for transplantation are described in greater detail in U.S. Patent Application Serial No. 08/220,741, filed March 31, 1994, the contents of which are incorporated herein by reference. The altered (also referred to herein as "modified") porcine cells can comprise an isolated population of cells. The characteristics of such populations are described above. The hepatocytes to be modified can be obtained from donor swine at the gestational ages described herein. Preferred donor swine are those which are essentially pathogen-free as described herein.

In another embodiment, the porcine hepatocytes of the present invention can be modified to inhibit natural antibody-mediated hyperacute rejection of the cells. For example, the hepatocytes of the invention may, in unmodified form, express an epitope on their surface which stimulates hyperacute rejection of the hepatocytes by natural antibodies in a recipient subject. Such an epitope can be altered, reduced or substantially eliminated. This treatment of the hepatocytes inhibits subsequent recognition of the epitope by natural antibodies in a recipient, thereby inhibiting hyperacute rejection. In a preferred embodiment, the epitope is a carbohydrate, preferably galactosyl(α1,3)galactose (Gal(α1,3)Gal). Dispersed cells can be treated or, alternatively, cells can be treated within a tissue or organ (e.g., liver).

Epitopes on the surface of the hepatocytes, in one embodiment of the invention, are removed from the surface of a cell, such as by enzymatic or chemical treatment of the cell. For example, Gal(α1,3)Gal epitopes can be cleaved from a cell surface by treatment of the cell with an alpha-galactosidase. In another embodiment, formation of the epitope on the cell surface is inhibited. This can be accomplished by inhibiting the activity of an enzyme which forms the epitope. For example, formation of Gal(α1,3)Gal epitopes on the surface of a cell can be interfered with by inhibiting the activity of an alpha-1,3-galactosyltransferase within the cell, such as by introducing into the cell a nucleic acid which is antisense to a coding or regulatory region of an alpha-1,3-galactosyltransferase gene or by treating the cell with a chemical inhibitor of the enzyme. In yet another embodiment, epitopes on a hepatocyte

surface are altered by binding a molecule to the epitope, thereby inhibiting its subsequent recognition by natural antibodies in a recipient. For example, lectins, antibodies or antibody fragments can be bound to an epitope to inhibit its subsequent recognition by natural antibodies. Methods for altering epitopes on cell surfaces which stimulate hyperacute rejection of the cells by natural antibodies in a recipient subject are described in greater detail in U.S. Patent Application Serial No. 08/253,782, filed June 3, 1994, the contents of which are incorporated herein by reference.

C. Porcine Hepatocytes and Isolated Populations of Porcine Hepatocytes Obtained from Essentially Pathogen-Free Swine

The invention also features a porcine hepatocyte (and an isolated population of porcine hepatocytes) obtained from a swine which is essentially free from organisms or substances which are capable of transmitting infection or disease to a xenogeneic recipient, e.g., a human recipient, of the cells. Typically, porcine hepatocytes are obtained from a swine which is essentially free from pathogens which affect humans. For example, the pathogens from which the swine are free include, but are not limited to, one or more of pathogens from the following categories of pathogens: parasites, bacteria, mycoplasma, and viruses. The swine can be free from, for example, parasites such as toxoplasma and eperytherozoon, or mycoplasma, such as M. hyopneumonia. Examples of bacteria from which the swine can be free include brucella, listeria, mycobacterium TB, leptospirillum, and haemophilus suis. Additionally, the swine can be free from viruses such as zoonotic viruses (viruses which can be transferred from pigs to man under natural conditions), viruses that can cross the placenta in pregnant sows, and neurotropic viruses. Zoonotic viruses include, for example, a virus in the rabies virus group, a herpes-like virus which causes pseudorabies, encephalomyocarditus virus, swine influenza Type A, transmissible gastroenteritus virus, parainfluenza virus 3 and vesicular stomatitis virus. Viruses that can cross the placenta include, for example, viruses that cause porcine respiratory reproductive syndrome, a virus in the rabies virus group, a herpes-like virus which causes pseudorabies, parvovirus, a virus that causes swine vesicular disease, techen (porcine polio virus), hemmaglutinating encephalomyocarditus, cytomegalovirus, suipoxvirus, and swine influenza type A. Neurotropic viruses include, for example, a virus in the rabies virus group, a herpes-like virus which causes pseudorabies, parvovirus, encephalomyocarditus virus, a virus which causes swine vesicular disease, porcine poliovirus (techen), a virus which causes hemmaglutinating encephalomyocarditus, adenovirus, parainfluenza 3 virus. Specific examples of viruses from which the swine are free include: a virus which causes (or results in) porcine respiratory reproductive syndrome, a virus in the rabies virus group, a herpes-like virus which causes pseudorabies, parvovirus, encephalomyocarditus virus, a virus which causes swine vesicular disease, porcine poliovirus (techen), a virus which causes hemmaglutinating encephalomyocarditus, cytomegalovirus, suipoxvirus, swine influenza type A, adenovirus,

transmissible gastroenteritus virus, a virus which causes bovine viral diarrhea, parainfluenza virus 3, and vesicular stomatitis virus.

In one embodiment, the pigs from which hepatocytes are isolated are essentially free from the following organisms: Toxoplasma, eperythrozoon, brucella, listeria, mycobacterium TB, leptospirillum, haemophilus suis, M. Hyopneumonia, a virus which causes porcine respiratory reproductive syndrome, a virus which causes rabies, a virus which causes pseudorabies, parvovirus, encephalomyocarditus virus, a virus which causes swine vesicular disease, porcine polio virus (techen), a virus which causes hemagglutinating encephalomyocarditus, suipoxvirus, swine influenza type A, adenovirus, transmissible gastroenteritis virus, a virus which causes bovine viral diarrhea, and vesicular stomatitis virus. The phrase "essentially free from organisms or substances which are capable of transmitting infection or disease to a xenogeneic recipient" (also referred to herein as "essentially pathogen-free") when referring to a swine from which cells are isolated means that that swine does not contain organisms or substances in an amount which transmits infection or disease to a xenogeneic recipient, e.g. a human. Example V provides representative, but not limiting, examples of methods for selecting swine which are essentially free from various pathogens. The hepatocytes of the invention can be isolated from embryonic or post-natal swine which are determined to be essentially free of such organisms. These swine are maintained under suitable conditions until used as a source of hepatocytes.

Preferred gestational ages of the swine from which these cells are obtained are described in detail herein. Porcine hepatocytes obtained from essentially pathogen-free swine can additionally be modified to reduce the immunogenicity of the cells following administration to a xenogeneic subject as described herein.

II. METHODS OF THE INVENTION

A. Methods of Treating Disorders Characterized by Insufficient Liver Function Using Porcine Hepatocytes

Still further aspects of the invention include methods for treating disorders characterized by insufficient liver function in a subject, particularly a human subject. These methods include administering to a xenogeneic subject, an isolated population of porcine hepatocytes described herein. The term "treating" as used herein includes reducing or alleviating at least one adverse effect or symptom of a disorder characterized by insufficient liver function. Non-limiting examples of adverse effects or symptoms of liver disorders include: high serum cholesterol and early onset atherosclerosis associated with familial hypercholesterolemia; absent glucuronyl transferase activity, impaired biliary excretion, severe unconjugated hyperbilirubinemia, and neurological damage associated with Crigler-Najjar Syndrome Type I; decreased glucuronyl transferase activity and unconjugated

hyperbilirubinemia associated with Gilbert's Syndrome; cirrhosis and liver failure associated with chronic hepatitis or other causes such as alcohol abuse; death in infancy associated with OTC deficiency; alveolar tissue damage associated with hereditary emphysema; deficiency in clotting factor VIII associated with hemophilia A. For additional examples of adverse effects or symptoms of a wide variety of liver disorders, see Robbins, S.L. et al. (1984) Pathological Basis of Disease (W.B. Saunders Company, Philadelphia) 884-942. Transplantation of porcine hepatocytes of the invention into a subject with a liver disorder results in replacement of lost or damaged hepatocytes and replacement of liver function. Porcine hepatocytes are transplanted into a subject with a liver disorder in an amount suitable to replace lost or damaged hepatocytes such that there is an at least partial reduction or alleviation of at least one adverse effect or symptom of the liver disorder.

As used herein the terms "administering", "introducing", and "transplanting" are used interchangeably and refer to the placement of the porcine hepatocytes of the invention into a subject, e.g., a xenogeneic subject, by a method or route which results in localization of the hepatocytes at a desired site. The porcine hepatocytes can be administered to a subject by any appropriate route which results in delivery of the cells to a desired location in the subject where at least a portion of the cells remain viable. It is preferred that at least about 5%, preferably at least about 10%, more preferably at least about 20%, yet more preferably at least about 30%, still more preferably at least about 40%, and most preferably at least about 50% or more of the cells remain viable after administration into a subject. The period of viability of the cells after administration to a subject can be as short as a few hours, e.g., twenty-four hours, to a few days, to as long as a few weeks to months. Common methods of administering hepatocytes to subjects, particularly human subjects, include intraperitoneal injection of the cells, (Wilson, J. et al. (1991) *Clin. Biotech.* 3(1):21-25), intravenous infusion of the cells into, for example, the portal vein (Kay, M. (1993) *Cell Trans.* 2:405-406; Tejera, J.L. et al. (1992) *Transplan. Proc.* 24(1):160-161; Wiederkehr, J.C. et al. (1990) *Transplantation* 50(3):466-476), or the mesenteric vein (Grossman, M. et al. (1994) *Nature Gen.* 6:335-341; Wilson, J.M. et al. (1990) *Proc. Natl. Acad. Sci.* 87:8437-8441), intrasplenic injection of the cells (Rhim, J.A. et al. (1994) *Science* 263:1149-1152; Kay, M.A. (1993) *Cell Trans.* 2:405-406; Wiederkehr, J.C. et al. (1990) *Transplantation* 50(3):466-476), and infusion of the cells into the splenic artery. To facilitate transplantation of the hepatocytes into, for example, the peritoneal cavity, the cells can bound to microcarrier beads such as collagen-coated dextran beads (Pharmacia, Uppsala, Sweden) (Wilson, J. et al. (1991) *Clin. Biotech.* 3(1):21-25). Cells can be administered in a pharmaceutically acceptable carrier or diluent as described herein. A human liver typically consists of about 2×10^{11} hepatocytes. To treat insufficient liver function in a human subject, about 10^9-10^{10} hepatocyes are transplanted into the subject. The whole liver of a pig weighing about fifty pounds yields about 10^{10} hepatocytes and provides, therefore, a sufficient number of hepatocytes for transplantation into a human subject.

To accomplish these methods of administration, the cells and/or compositions of the invention can be inserted into a delivery device which facilitates introduction of the cells and/or compositions into the subject. Such delivery devices include tubes, e.g., catheters, for infusing or injecting cells and fluids into the body of a recipient subject. In a preferred embodiment, the tubes additionally have a needle, e.g., a syringe, through which the cells of the invention can be introduced into the subject at a desired location. The porcine hepatocytes (and compositions containing the hepatocytes) of the invention can be inserted into such a delivery device, e.g., a syringe, e.g., syringe pump, in different forms. For example, the cells can be suspended in a solution or embedded in a support matrix when contained in such a delivery device. As used herein, the term "solution" includes a pharmaceutically acceptable carrier or diluent in which the cells of the invention remain viable. Pharmaceutically acceptable carriers and diluents include sterile saline and aqueous buffer solutions. The use of such carriers and diluents is well known in the art. The solution is preferably sterile and fluid to the extent that easy syringability exists. Preferably, the solution is stable under the conditions of manufacture and storage and preserved against the contaminating action of microorganisms such as bacteria and fungi through the use of, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. Solutions of the invention can be prepared by incorporating porcine hepatocytes as described herein in a pharmaceutically acceptable carrier or diluent and, as required, other ingredients enumerated above, followed by filtered sterilization.

Support matrices in which the porcine hepatocytes can be incorporated or embedded include matrices which are recipient-compatible and which degrade into products which are not harmful to the recipient. Natural and/or synthetic biodegradable matrices are examples of such matrices. Natural biodegradable matrices include collagen matrices. Synthetic biodegradable matrices include synthetic polymers such as polyanhydrides, polyorthoesters, and polylactic acid. These matrices provide support and protection for the hepatocytes *in vivo*.

The term "subject" as used herein refers to mammals, particularly humans, susceptible to disorders characterized by insufficient liver function. The term "subject" also includes mammals in which an immune response is elicited against allogeneic or xenogeneic cells. Examples of subjects include primates (e.g., humans, and monkeys). A "xenogeneic subject" (also referred to herein as "recipient subject" or "recipient") as used herein is a subject into which cells of another species are introduced or are to be introduced.

As used herein, the language "disorder characterized by insufficient liver function" includes a disorder in which there is abnormal liver function. Abnormal liver function includes an impairment or absence of a normal liver function or presence of an abnormal liver function. Abnormal liver function can result from a genetic disorder involving, for example, a cell surface protein. An example of such a disorder is familial hypercholesterolemia which is characterized by a low expression of the low density lipoprotein receptor. Abnormal liver

function can also result from a genetic disorder involving a liver enzyme. Examples of genetic disorders involving a liver enzyme include Crigler-Najjar Syndrome Type I and Type II, OTC deficiency, and phenylketonuria. In Crigler-Najjar Syndrome Type I, infants lack the enzyme UDP-glucuronyl transferase which is necessary to conjugate and excrete bilirubin, the breakdown product of heme from red blood cells. The toxic metabolites that accumulate as a result of this Syndrome cause fatal neurological damage. OTC deficiency is caused by a lack of the urea cycle enzyme, ornithine transcarbamoylase. This deficiency results in death in infancy. Phenylketonuria results from a lack of the liver enzyme phenylalanine hydroxylase. If untreated, lack of phenylalanine hydroxylase leads to hyperphenylalaninemia and usually mental retardation.

Alternatively, abnormal liver function can result from a genetic disorder involving secreted proteins. Hereditary emphysema, for example, is a genetic disorder occurring in one in 2,000 individuals in which there is a mutation in a secreted protein, alpha-1-antitrypsin (AAT). In the absence of functional AAT, neutrophil elastase, released during lung inflammation, proceeds unopposed to damage alveolar tissue. Hemophilias are bleeding disorders resulting from genetic defects in several of the clotting factors that are secreted by the liver. Hemophilia A, for example, is caused by a deficiency in Factor VIII, a clotting factor secreted by hepatocytes. These genetic disorders all result from the inability of the liver to produce (and, in some instances) secrete various proteins which it normally produces or to produce various proteins at the level which they are normally produced.

Abnormal liver function can result from a variety of non-genetic disorders that cause acute liver failure or that lead to chronic liver failure. Acute liver failure can result from a number of different causes such as drug or toxin ingestion, viral infection, and metabolic disease. Drugs which have been associated with acute liver failure include halothane, isoniazid, α-methyldopa, acetaminophen, and iproniazid. Viral infection, such as by hepatitis A virus, hepatitis B virus, or the group non-A, non-B hepatitis viruses, can also be a cause of acute liver failure. Chronic liver failure is most often caused by alcoholic cirrhosis and chronic active hepatitis. Hepatitis B and the non-A, non-B hepatitis viruses are the viruses most often associated with chronic liver failure. The methods of the invention can result in alleviation or reduction of any one or a combination of the adverse effects or symptoms of liver disorders described herein and/or which are known in the art.

Prior to introduction into a subject, the porcine hepatocytes can be modified to inhibit immunological rejection. The porcine hepatocytes can, as described in detail herein, be rendered suitable for introduction into a xenogeneic subject by alteration of at least one immunogenic cell surface antigen (e.g., an MHC class I antigen). To inhibit rejection of transplanted porcine hepatocytes and to achieve immunological non-responsiveness in an allogeneic or xenogeneic transplant recipient, the method of the invention can include alteration of immunogenic antigens on the surface of the porcine hepatocytes prior to introduction into the subject. This step of altering one or more immunogenic antigens on

porcine hepatocytes can be performed alone or in combination with administering to the subject an agent which inhibits T cell activity in the subject. Alternatively, inhibition of rejection of a porcine hepatocyte graft can be accomplished by administering to the subject an agent which inhibits T cell activity in the subject in the absence of prior alteration of an immunogenic antigen on the surface of the porcine cardiomyocytes. As used herein, an agent which inhibits T cell activity is defined as an agent which results in removal (e.g., sequestration) or destruction of T cells within a subject or inhibits T cell functions within the subject (i.e., T cells may still be present in the subject but are in a non-functional state, such that they are unable to proliferate or elicit or perform effector functions, e.g. cytokine production, cytotoxicity etc.). The term "T cell" encompasses mature peripheral blood T lymphocytes. The agent which inhibits T cell activity may also inhibit the activity or maturation of immature T cells (e.g., thymocytes).

A preferred agent for use in inhibiting T cell activity in a recipient subject is an immunosuppressive drug. The term "immunosuppressive drug or agent" is intended to include pharmaceutical agents which inhibit or interfere with normal immune function. A preferred immunsuppressive drug is cyclosporin A. Other immunosuppressive drugs which can be used include FK506, RS-61443, and deoxyspergualin. In one embodiment, the immunosuppressive drug is administered in conjunction with at least one other therapeutic agent. Additional therapeutic agents which can be administered include steroids (e.g., glucocorticoids such as prednisone, methyl prednisolone and dexamethasone) and chemotherapeutic agents (e.g., azathioprine and cyclosphosphamide). In another embodiment, an immunosuppressive drug is administered in conjunction with both a steroid and a chemotherapeutic agent. Suitable immunosuppressive drugs are commercially available (e.g., cyclosporin A is available from Sandoz, Corp., East Hanover, NJ).

An immunsuppressive drug is administered in a formulation which is compatible with the route of administration. Suitable routes of administration include intravenous injection (either as a single infusion, multiple infusions or as an intravenous drip over time), intraperitoneal injection, intramuscular injection and oral administration. For intravenous injection, the drug can be dissolved in a physiologically acceptable carrier or diluent (e.g., a buffered saline solution) which is sterile and allows for syringability. Dispersions of drugs can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Convenient routes of administration and carriers for immunsuppressive drugs are known in the art. For example, cyclosporin A can be administered intravenously in a saline solution, or orally, intraperitoneally or intramuscularly in olive oil or other suitable carrier or diluent.

An immunosuppressive drug is administered to a recipient subject at a dosage sufficient to achieve the desired therapeutic effect (e.g., inhibition of rejection of transplanted cells). Dosage ranges for immunosuppressive drugs, and other agents which can be coadministered therewith (e.g., steroids and chemotherapeutic agents), are known in the art (*See e.g.*, Freed et al. *New Engl. J. Med.* (1992) 327:1549: Spencer et al. (1992) *New Engl. J.*

Med. 327:1541; Widner et al. (1992) *New Engl. J. Med.* 327:1556; Lindvall et al. (1992) *Ann. Neurol.* 31:155; and Lindvall et al. (1992) *Arch. Neurol.* 46:615). A preferred dosage range for immunosuppressive drugs, suitable for treatment of humans, is about 1-30 mg/kg of body weight per day. A preferred dosage range for cyclosporin A is about 1-10 mg/kg of body weight per day, more preferably about 1-5 mg/kg of body weight per day. Dosages can be adjusted to maintain an optimal level of the immunosuppressive drug in the serum of the recipient subject. For example, dosages can be adjusted to maintain a preferred serum level for cyclosporin A in a human subject of about 100-200 ng/ml. It is to be noted that dosage values may vary according to factors such as the disease state, age, sex, and weight of the individual. Dosage regimens may be adjusted over time to provide the optimum therapeutic response according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that the dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition.

In one embodiment of the invention, an immunsuppressive drug is administered to a subject transiently for a sufficient time to induce tolerance to the transplanted cells in the subject. Transient administration of an immunosuppressive drug has been found to induce long-term graft-specific tolerance in a graft recipient (*See* Brunson et al. (1991) *Transplantation* 52:545; Hutchinson et al. (1981) *Transplantation* 32:210; Green et al. (1979) *Lancet* 2:123; Hall et al. (1985) *J. Exp. Med.* 162:1683). Administration of the drug to the subject can begin prior to transplantation of the cells into the subject. For example, initiation of drug administration can be a few days (e.g., one to three days) before transplantation. Alternatively, drug administration can begin the day of transplantation or a few days (generally not more than three days) after transplantation. Administration of the drug is continued for sufficient time to induce donor cell-specific tolerance in the recipient such that donor cells will continue to be accepted by the recipient when drug administration ceases. For example, the drug can be administered for as short as three days or as long as three months following transplantation. Typically, the drug is administered for at least one week but not more than one month following transplantation. Induction of tolerance to the transplanted cells in a subject is indicated by the continued acceptance of the transplanted cells after administration of the immunosuppressive drug has ceased. Acceptance of transplanted tissue can be determined morphologically (e.g., with biopsies of liver) or by assessment of the functional activity of the graft.

Another type of agent which can be used to inhibit T cell activity in a subject is an antibody, or fragment or derivative thereof, which depletes or sequesters T cells in a recipient. Antibodies which are capable of depleting or sequestering T cells *in vivo* when administered to a subject are known in the art. Typically, these antibodies bind to an antigen on the surface of a T cell. Polyclonal antisera can be used, for example anti-lymphocyte serum. Alternatively, one or more monoclonal antibodies can be used. Preferred T cell-

depleting antibodies include monoclonal antibodies which bind to CD2, CD3, CD4 or CD8 on the surface of T cells. Antibodies which bind to these antigens are known in the art and are commercially available (e.g., from American Type Culture Collection). A preferred monoclonal antibody for binding to CD3 on human T cells is OKT3 (ATCC CRL 8001). The binding of an antibody to surface antigens on a T cell can facilitate sequestration of T cells in a subject and/or destruction of T cells in a subject by endogenous mechanisms. Alternatively, a T cell-depleting antibody which binds to an antigen on a T cell surface can be conjugated to a toxin (e.g., ricin) or other cytotoxic molecule (e.g., a radioactive isotope) to facilitate destruction of T cells upon binding of the antibody to the T cells. *See* U.S. Patent Application Serial No.: 08/220,724, filed March 31, 1994, for further details concerning the generation of antibodies which can be used in the present invention.

Another type of antibody which can be used to inhibit T cell activity in a recipient subject is an antibody which inhibits T cell proliferation. For example, an antibody directed against a T cell growth factor, such as IL-2, or a T cell growth factor receptor, such as the IL-2 receptor, can inhibit proliferation of T cells (*See e.g.*, DeSilva, D.R. et al. (1991) *J. Immunol.* 147:3261-3267). Accordingly, an IL-2 or an IL-2 receptor antibody can be administered to a recipient to inhibit rejection of a transplanted cell (see e.g. Wood et al. (1992) *Neuroscience* 49:410). Additionally, both an IL-2 and an IL-2 receptor antibody can be coadministered to inhibit T cell activity or can be administered with another antibody (e.g., which binds to a surface antigen on T cells).

An antibody which depletes, sequesters or inhibits T cells within a recipient can be administered at a dose and for an appropriate time to inhibit rejection of cells upon transplantation. Antibodies are preferably administered intravenously in a pharmaceutically acceptable carrier or diluent (e.g., a sterile saline solution). Antibody administration can begin prior to transplantation (e.g., one to five days prior to transplantation) and can continue on a daily basis after transplantation to achieve the desired effect (e.g., up to fourteen days after transplantation). A preferred dosage range for administration of an antibody to a human subject is about 0.1-0.3 mg/kg of body weight per day. Alternatively, a single high dose of antibody (e.g., a bolus at a dosage of about 10 mg/kg of body weight) can be administered to a human subject on the day of introduction of the hepatocytes into the subject. The effectiveness of antibody treatment in depleting T cells from the peripheral blood can be determined by comparing T cell counts in blood samples taken from the subject before and after antibody treatment. Dosage regimes can be adjusted over time to provide the optimum therapeutic response according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions. Dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition.

To assess their therapeutic potential in humans, the porcine hepatocytes of the invention can be introduced into existing animal models for, for example, familial

hypercholesterolemia, Crigler-Najjar Syndrome Type I, OTC deficiency, and acute liver failure. The Watanabe heritable hyperlipidemic (WHHL) rabbit, an animal model for homozygous familial hypercholesterolemia, has a mutation in the low density lipoprotein (LDL) receptor that results in defective clearance of LDL and accumulation of cholesterol-rich lipoproteins in plasma. Brown, M.S. et al. (1986) *Science* 232:34-47; Havel, R.J. et al. (1989) *Arteriosclerosis Supplement* 9:I-33-I-38; Yamamoto, T. et al. (1986) *Science* 232:1230-1237. In addition, the Gunn rat, which lacks the enzyme UDP-glucuronyl transferase, which is necessary to conjugate and excrete bilirubin, the breakdown product of heme from red blood cells, is an animal model of Crigler-Najjar Syndrome Type I. Dixit, W. et al. (1993) *Transplantation* 55:616-622. Toxic metabolites that accumulate in the absence of this enzyme can cause fatal neurological damage. A mouse model of OTC deficiency is also available. Morsy, M.A. et al. (1993) *J. Clin. Invest.* 92:1580-1586. Treatment of mice and rats with the selective hepatotoxin, D-galactosamine, results in necrosis of the hepatic parenchyma and death due to acute liver failure. This model of acute liver failure is described in Shinozuka, H. et al. (1973) *Fed. Proc.* 32:1516-1526.

The therapeutic efficacy of the administered porcine hepatocytes in the WHHL rabbit is typically determined by, for example, measurement of serum cholesterol levels and clearance of radioactive LDL as described in the Examples section. Normalization of serum hypercholesterolemia demonstrates that the administered porcine hepatocytes can be used to treat familial hypercholesterolemia. The efficacy of the administered porcine hepatocyes in the Gunn rat is measured by total serum bilirubin levels or conjugated bilirubin levels. The efficacy of the administered porcine hepatocytes in the OTC-deficient mouse model can be measured by increased levels of OTC as described, for example, in Grompe, M. et al. (1992) *Hum. Gene Therapy* 3:35. Other methods of determining the therapeutic potential are histological examination of the hepatocyte graft (via a biopsy), e.g., by staining for, for example, the presence of an enzyme not produced in the recipient subject but provided by the administered porcine hepatocytes. In the case of acute hepatic failure, hepatocyte transplantation is especially suited for short term provision of, for example, metabolic function in a subject waiting for a whole liver transplant or in a subject with acute liver damage, e.g., caused by viral hepatitis, while the subject's liver is regenerating after removal of the damage-causing agent. Chronic liver failure induced by, for example, carbon tetrachloride, can also be treated by hepatocyte transplantation.

This invention is further illustrated by the following examples which in no way should be construed as being further limiting. The contents of all cited references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference.

EXAMPLES

Example I: Isolation of Porcine Hepatocytes

Male Yorkshire outbred pigs (20-30 kg) were obtained from the Tufts Veterinary Facility in Grafton, MA. The pigs were sacrificed and the left lateral lobe was mobilized, clamped and excised. The lobe was perfused with cold PBS (1 liter). A second perfusion (500 ml) with ViaSpan (Belzer UW) (Dupont, Wilmington, DE) was performed prior to transport to from Tufts Veterinary Facility to the transplant facilities. Liver lobes were transported on ice in ViaSpan.

Porcine hepatocytes were isolated by the two stage perfusion technique originally described by Berry and Friend ((1969) *J. Cell Biol.* 43:506-520) and modified by others (Maganto P. et al. (1992) *Transplant Proc.* 24:2826-2827; Gerlach J.C. et al. (1994) *Transplantation* 57:1318-1322) for *ex vivo* perfusion of large animal organs. A liver lobe of 100-200 g was cannulated and perfused with HBSS (minus Mg^{++}, Ca^{++}) containing 0.4 mM EDTA, 10 mM HEPES, pH 7.4 and penicillin (100 U/ml)-streptomycin (100 ug/ml) at 35°C. This was followed by a second perfusion with complete HBSS containing collagenase P (0.8 mg/ml, Boehringer Mannheim), 10 mM HEPES, pH 7.4, and penicillin-streptomycin at 35°C. The perfusion was continued until visible softening of the organ had occurred. The total time for digestion ranged from 12- 20 minutes. The digested liver was then physically disrupted and the released hepatocytes were washed (50 x g) twice in DMEM/Weymouth media containing 10% heat inactivated calf serum at 4°C.

Porcine hepatocytes were collected and counted. Viability was assessed by trypan blue staining and was routinely greater than 88%. Mean cell yields were 2.1 +/-1 x 10^7/gram wet weight. The purity of the hepatocyte preparation was judged to be over 98% by immunofluorescence for class II bearing non-parenchymal cells. Purity determinations were made by counting the positive staining cells (monoclonal antibody ISCR3) in several fields consisting of 200 cells. Cells were stored in cold (4°C) HBSS or ViaSpan transport medium up to 5 hours prior to transplantation. Immediately prior to infusion the hepatocytes were centrifuged at 50 x g for 5 minutes. The hepatocytes were gently resuspended in 20 ml HBSS containing penicillin/streptomycin or gentamycin and 10 units/ml heparin at 1 x 10^7 cells/ml.

Example II: Transplantation of Porcine Hepatocytes into Xenogeneic Recipients and Demonstration of Hepatocyte Survival *In Vivo*

The Watanabe heritable hyperlipidemic (WHHL) rabbit, an animal model for homozygous familial hypercholesterolemia (FH), has a mutation in the low density lipoprotein (LDL) receptor that results in defective clearance of LDL and accumulation of cholesterol-rich lipoproteins in plasma. WHHL rabbits were purchased from CAMM

Research (Wayne, NJ). The rabbits received standard rabbit chow and water *ad libitum*. WHHL rabbits (2-3 kg) were anesthetized with xylazine and ketamine and maintained under isoflurane. An incision distal and parallel to the end of the rib cage was made. The peritoneum was incised and the portal vein exposed. Hepatocytes (2×10^8) suspended in 20 ml of HBSS (20°C) were infused into the portal vein via a 25 gauge syringe connected to a Baxter model A infusion pump at approximately 1 ml/min. Portal hypertension was not monitored, but higher infusion rates resulted in significant mortality presumably due to portal occlusion. The injection site was covered with gel foam to prevent leakage as needed. Using this protocol, surgical mortality was minimized (below 13%). Cyclosporin A (Sandimmune, Sandoz) was administered daily at 10 mg/kg (s.c.) starting on the day of surgery.

The serum from WHHL rabbits was subjected to immunoprecipitation using an anti-porcine albumin antibody (Research Plus, Bayonne, NJ) coupled to CNBr activated Sepharose 4B (Pharmacia). To eliminate cross reactivity with rabbit albumin the anti-porcine albumin antiserum was preabsorbed over an affinity column prepared by coupling rabbit albumin to CNBr Sepharose 4B. Serum samples (50 µl) were diluted in PBS containing 0.2% Tween-20 to a total reaction volume of 250 µl. Samples were incubated for 20 hours at 4°C with gentle rocking. Beads were centrifuged and washed in PBS-Tween-20 prior to final resuspension in gel loading buffer. Samples were run on 8% SDS-polyacrylamide gels. Gels were electophoretically transferred to nitrocellulose and probed with a goat anti-porcine albumin antibody (Bethyl Laboratories, Montgomery, TX). The immunoblots were incubated with horseradish peroxidase conjugated anti-goat IgG . The immune complexes were visualized by ECL (Amersham Life Sciences). Pretransplant bleeds and commercial porcine albumin were used as controls. The antibody used does not detect rabbit serum albumin as shown in the Western blot of the pretransplant serum. The results of this experiment are illustrated in Figure 1. The production of porcine albumin confirmed the presence of viable functioning hepatocytes in the graft. Serum samples indicated the presence of porcine albumin 6 weeks after transplantation, the last time point assayed.

To assess the secretion of porcine albumin in all the rabbits, a capture ELISA was developed for porcine albumin. The capture ELISA was developed using the rabbit (Research Plus) anti-porcine albumin antibody coated on 96 well microtiter plates. Rabbit serum (1:10 diluted) was added to the plate and after washing and binding of the goat anti-porcine albumin antibody (Bethyl Laboratories), detection was carried out with horseradish peroxidase conjugated anti-goat IgG (Jackson ImmunoResearch, West Grove, PA) and o-phenylenediamine. The sensitivity of this assay was in the nanogram range. Porcine albumin was assayed in animals at 2 weeks and 1 month post surgery. The results are shown in Table I. Rabbit serum was collected at the times indicated and analyzed using the capture assay. All transplanted animals showed albumin production in the µg/ml range with the exception of R3728. Sham surgery animals (R3535) were consistently negative for porcine albumin production.

TABLE I

Rabbit ID Number	Porcine Albumin (A_{490}) at Seven Days Post-Transplant	Porcine Albumin (A_{490}) at Thirty Days Post-Transplant
Transplanted Rabbits		
R3530[1]	1.08	0.48
R3533	0.98	0.66
R3736	1.33	0.64
R3738	0.81	0.54
R4045	0.91	0.09
R3744	0.15	0.04
R3745	0.21	0.07
Control Rabbit		
R3739[2]	0.00	0.00

[1] All animals received cyclosporin at 10 mg/kg/day. One animal (R3728) did not exhibit detectable porcine albumin in this assay.

[2] Sham surgery control.

The presence of porcine hepatocytes in the WHHL rabbits was confirmed at autopsy by immunohistochemistry. Liver biopsies were fixed in 10% formalin and embedded in paraffin. Sections (5μm) were prepared and stained with hematoxylin and eosin. Detection of secreted porcine albumin was accomplished using a specific anti-porcine albumin antibody. Antibody binding was visualized by a biotin-streptavidin peroxidase system (Biogenex) using AEC as the chromagen. Sections were taken randomly from various regions of the liver lobes. Negative controls were developed in the absence of primary antibody using adjacent tissue sections. Paraffin sections of transplanted WHHL rabbits were fixed and stained with a porcine specific anti-albumin IgG. The results of this experiment are illustrated in Figures 2A-2F. Figures 2A and 2B show the control tissues for pig and non-transplanted WHHL rabbit respectively. Figures 2C to 2F represent a time course following transplantation of porcine hepatocytes into the parenchyma of the recipient lobe. Figure 2C is 1 hour post surgery. Figures 2D and 2E are 4 days after surgery. Figure 2F is at 7 months after surgery. At the time of surgery the porcine cells are contained within the hepatic sinusoids (Figure 2C). Two days after transplantation (Figure 2D) the porcine hepatocytes can be seen adhering to and migrating into the endothelial lining of the vessels and adjacent parenchyma. After the graft has been established as indicated by long term lowering of serum cholesterol (Figure 2E, R3530) numerous foci of cells are seen dispersed within the liver parenchyma where they appear to have integrated among the host cells.

Example III: Transplantation of Porcine Hepatocytes into Xenogeneic Recipients and Demonstration of Hepatocyte Function *In Vivo*

Pre- and post-operative rabbit serum was collected from non-fasted animals using a morning bleed time for consistency. The serum was stored frozen. All sera were assayed for total serum cholesterol at a 1:1 or 1:2 dilution with physiologic saline by the cholesterol oxidase procedure. (Sigma Diagnostics, St. Louis, MO). Interassay variations for the cholesterol assay were 10-20%. Intra-assay variation was less then 10%. Reductions in serum cholesterol were noted within the first few weeks following transplantation. The results of this experiment are illustrated in Figure 3. Rabbits were infused with 1-2 x 10^8 porcine hepatocytes via the portal vein. Total serum cholesterol levels were determined as described above. Pretransplant levels were: for rabbit R3738, 535±49 mg/dl; for rabbit R3779, 690±24 mg/dl; for rabbit R3777, 610±24 mg/dl; and for rabbit R4045, 800±26 mg/dl; R3728, 560±41 mg/dl. Rabbit R3739 was a sham surgery control with carrier only infused. Rabbit R3534 was a control rabbit that did not receive cyclosporin. All other rabbits were injected with cyclosporin (s.c.) at 10 mg/kg/day.

As shown in Figure 3, reductions in total serum cholesterol reached 60-65% of pretransplant levels. All recipient animals achieved a 30-40% reduction within the first 10-15 days after transplantation. The mean serum cholesterol reduction for animals under immunosuppression remained at 40.4 +/- 5.6% of pretransplant levels for the 3-8 weeks after transplantation. An animal that was infused with porcine hepatocytes in the absence of cyclosporin administration (R3534) showed no sustained decrease in serum cholesterol. Cholesterol levels in a sham surgery animal (no cells infused) were reduced by a maximum of 10-20% over the same time interval. One rabbit transplanted with porcine hepatocytes showed no decrease in serum cholesterol.

Three WHHL rabbits were transplanted and monitored for longer time periods to determine the duration of serum cholesterol lowering. The results of this experiment are illustrated in Figure 4. All animals received cyclosporin at 10 mg/kg/day. Total serum cholesterol values were determined as a function of time post-transplantation. Pretransplant values were as follows: for rabbit R3530, 635±27 mg/dl; for rabbit R3533, 730±20 mg/dl; and for rabbit R3736, 580±40 mg/dl; R3739, 615 mg/dl. As shown in Figure 4, the decrease in serum cholesterol levels was sustained for 3 months following hepatocellular transplantation. Two of these animals (R3530, R3533) demonstrated maximum decreases in total serum cholesterol of 60%-70%.

To confirm that the decrease in serum cholesterol was attributable to the transplanted porcine hepatocytes, a rabbit was removed from immunosuppression 30 days after transplantation. The results of this experiment are illustrated in Figure 5. Animals had been treated with cyclosporin A at 10 mg/kg/day starting the day after transplantation. The arrow

indicates the time after transplantation that the animal was taken off cyclosporin A treatment. As shown in Figure 5, the cessation of cyclosporin administration resulted in a return of total serum cholesterol to pretransplant levels suggesting that rejection of the cells caused an increase in serum cholesterol. The increase in serum cholesterol values was apparent within the first 10 days of withdrawal of the drug. Also shown in Figure 5 is a control rabbit that had not received cyclosporin in conjunction with the hepatocyte transplant. Rabbits receiving cyclosporin but no porcine hepatocytes showed no sustained reduction in serum cholesterol. Further decreases in serum cholesterol of WHHL rabbits could be achieved on reinfusion of porcine hepatocytes. The serum cholesterol levels in two WHHL rabbits (R3777, R3779) were decreased by approximately 40% over an initial 30 day time period after transplantation with porcine hepatocytes. In contrast to the rabbits shown in Figure 3, these animals showed an increase in serum cholesterol starting at day 20-30. To determine whether further decreases in serum cholesterol could be achieved, a second infusion of 2×10^8 porcine hepatocytes was completed 78 days after the initial transplant. Both rabbits showed an immediate lowering of cholesterol levels (Figure 6) that was sustained until at least 100 days after the initial transplant. A third animal, R3535, that had not been previously transplanted with porcine cells, was also infused and also showed an immediate lowering of cholesterol levels that was sustained 100 days after transplant. All animals received cyclosporin.

To establish that the transplanted hepatocytes were functional and were responsible for the noted reductions in cholesterol levels, clearance studies were performed using iodinated human LDL. Labeled human LDL (20-40 μCi) was delivered via the marginal ear vein in physiological saline containing 2 mg/ml bovine serum albumin. Blood was collected from the opposite ear at intervals after injection. ^{125}I-Apolipoprotein B-containing LDL was precipitated with isopropanol (Holmquist, L. et al. (1978) *Anal. Biochem.* 88:457-460; Lagrost, L. et al. (1989) *J. Lipid Res.* 30:701-710). Percent clearance from the plasma was determined from duplicate counts taking the 2 minute time point as 100%. The animals were not starved prior to this study. The experiments were designed to measure the differences in clearance rates of the diseased control (nontransplanted WHHL rabbit), normal control New Zealand White and transplanted WHHL rabbits. The results of this experiment are illustrated in Figure 7. The WHHL rabbit transplanted with porcine hepatocytes showed a significant increase in the rate of LDL clearance relative to the nontransplanted WHHL rabbit. The time required for clearance of 50% of the serum ^{125}I-hLDL was 10.5 hr for a control WHHL rabbit, 6 hours for a transplanted WHHL rabbit and 2.5 hours for a New Zealand White rabbit.

To determine the effect of hepatocellular transplantation on the relative levels of HDL, LDL/IDL and VLDL in the recipient animal, FPLC fractionation was undertaken. Serum samples (200 μl) from New Zealand White, pretransplant WHHL rabbit and post-transplant WHHL rabbit were fractionated by FPLC on a Superose HR6 column. (Pharmacia) in a buffer containing 0.15M NaCl, 0.01% EDTA, 0.02% NaN_3, 0.01M Tris-

HCl, pH 7.4 (22,23). HDL and LDL peaks were identified using purified commercial lipoproteins (Organon Teknika, Rockville, MD). The column was run at 0.4 ml/min. Fractions (0.6 ml) were collected and assayed for total serum cholesterol after lyophilization. The results of this experiment are illustrated in Figure 8. The results in Figure 8 show the lipoprotein profile of a WHHL rabbit at intervals after hepatocellular transplantation. A distinct lowering of the LDL/IDL-cholesterol level was observed with time, consistent with the decreases in total serum cholesterol. Decreases in cholesterol were also observed in the region of VLDL, presumably due to increased conversion to IDL/LDL and subsequent uptake. WHHL rabbits are characterized by low levels of serum HDL. This fraction was not detectable prior to transplantation but following porcine hepatocyte transplantation an HDL-cholesterol fraction became detectable (see inset Figure 8)

Example IV: Transplantation of Masked Porcine Hepatocytes into Xenogeneic Recipients and Demonstration of Hepatocyte Function *In Vivo*

To demonstrate that porcine hepatocytes which are modified by binding anti-MHC class I antibodies to the MHC class I antigens on their surface survive and function in a xenogeneic subject, porcine hepatocytes were incubated with $F(ab')_2$ fragments of PT-85, a mouse monoclonal antibody specific for porcine MHC class I. This incubation was performed in PBS for 1 hour at 4°C with 1 μg antibody/10^6 cells. The hepatocytes having these antibodies bound to their MHC class I surface antigens are referred to hereinafter as "masked" hepatocytes. Control cells (unmasked) were incubated for the same time period in PBS or with a control $F(ab')_2$ fragment prepared from a monoclonal antibody (10-14) that binds to porcine CD-44. Prior to transplantation the cells were washed in Hanks solution at 4°C to remove unbound antibody, and control cells were treated in the same way.

WHHL rabbits were transplanted with 2 x 10^8 masked porcine hepatocytes by portal vein infusion. Total serum cholesterol levels were determined as described in Example III. Pretransplant levels were: for rabbit R3531, 715 mg/dl; and for rabbit R3532, 670 mg/dl; R-4138, 970 mg/dl. Rabbit 4054 was a control animal that received porcine hepatocytes masked with 10-14, an irrelevant antibody. The results of this experiment are illustrated in Figure 9. As shown in Figure 9 reductions in total serum cholesterol were observed and these reductions were maintained for 80 days. Unmasked cells or cells incubated with control monoclonal antibody were rejected as manifested by a return of serum cholesterol to pre-transplant levels within two weeks after transplantation. Two animals that were transplanted with masked cells did not show reduced serum cholesterol levels; this may be associated with limitations of the surgical procedure. Several rabbits were treated with subtherapeutic cyclosporin for two weeks in conjunction with masking. Total serum cholesterol levels were determined as described in Example III. Pretransplant levels were: for rabbit R3741, 975

mg/dl; for rabbit R4046, 900 mg/dl; for rabbit R4139, 850 mg/dl; and for rabbit R4246, mg/dl. Two rabbits were injected with cyclosporin (s.c.) at 10 mg/kg/day for 14 days (ST) in combination with masking and two rabbits were injected with cyclosporin (s.c.) at 10 mg/kg/day for just one day (SD). The results of this experiment are illustrated in Figure 10. As shown in Figure 10, serum cholesterol levels of all four rabbits decreased. Again reductions in serum cholesterol were observed in the animals receiving masked cells. Five of eight animals treated with masked cells in combination with short term cyclosporin showed sustained serum cholesterol reductions of over 20%.

Clearance studies using iodinated human LDL were performed to determine whether the cholesterol reductions were attributable to increased LDL uptake. Intact LDL-cholesterol was precipitated from serum using isopropanol at intervals after the injection of the radiolabelled LDL. The results of this experiment are illustrated in Figure 11. Rabbits were injected with ^{125}I-hLDL at time 0. The time course of clearance was monitored following isopropanol precipitation of 0.5 ml of plasma with 100% defined as the ^{125}Icpm precipitated at 3 minute post injection. The WHHL rabbit transplanted with masked porcine hepatocytes showed a significant increase in the rate of LDL clearance relative to the nontransplanted WHHL rabbit. The time required for clearance of 50% of the serum ^{125}I-LDL was 21 hours for a control WHHL rabbit, 11 hours for a transplanted WHHL rabbit and 4 hours for a New Zealand White rabbit. This represents a two fold increase in the rate of LDL clearance in the WHHL rabbit transplanted with masked hepatocytes and indicates that the masked porcine cells remained functional and were responsible for the reduced serum cholesterol.

To determine the effect of hepatocellular transplantation on the relative levels of HDL, LDL/IDL and VLDL in the recipient animal, FPLC fractionation was undertaken. The results of this experiment are illustrated in Figure 12. The lipoprotein profile of a WHHL rabbit at intervals after transplantation of masked hepatocytes is shown in Figure 12. A distinct lowering of the LDL/IDL-cholesterol level was observed with time, consistent with the decreases in total serum cholesterol. Decreases in cholesterol were also observed in the region of VLDL, presumably due to increased conversion to IDL/LDL and subsequent uptake.

The transplanted masked porcine hepatocytes were detected in paraffin sections of WHHL rabbit liver by *in situ* hybridization using a probe that hybridizes with a pig specific repetitive element present in numerous copies in the pig genome. This 234 base pair fragment of DNA is specific for hybridization with pig DNA. Digoxygenin-labeled probe was hybridized to paraffin sections and staining was accomplished by incubation with alkaline phosphatase labeled antidigoxygenin antibody and 5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt/nitro-blue tetrazolium chloride. The results of this experiment are illustrated in Figures 13A-13D. Figure 13A shows control rabbit liver. Figure 13B shows a pig liver hybridized with the probe. Figure 13C (low magnification) and Figure 13D (high magnification) are from a WHHL rabbit transplanted with masked porcine

hepatocytes. After the graft had been established as indicated by long term lowering of serum cholesterol, numerous foci of cells were seen dispersed within the liver parenchyma where they appeared to have integrated among the host cells (Figures 13C and 13D).

As an additional way of determining whether porcine cells were present in the rabbit livers, a polymerase chain reaction (PCR)-based method of detection was developed using primers for the 234 base pair porcine repetitive element. Oettinger et al. (1995) *Cell Trans.* 4:235. Rabbit livers were frozen on dry ice and stored at -80°C. The tissue was thawed, cut into 2 mm^3 sections and digested with proteinase K for 18 hours at 65°C. After precipitation of protein the DNA was extracted. Samples containing 1 μg DNA were amplified by PCR using AmpliTaq (Perkin Elmer) for 30 cycles. The products were electrophoresed on a 2% agarose gel. The results shown in Figure 14 demonstrate that pig cells were present in the transplanted livers. Pig specific bands are seen in 4 of 6 tissue sections from the WHHL rabbit (R-3532, six months post transplant). A non-transplanted rabbit liver (R) shows no band. Pig liver is the positive control.

Example V: Methods of Producing Essentially Pathogen-Free Swine from which Hepatocytes of the Invention can be Obtained

A. Collecting, processing, and analyzing pig fecal samples for signs of pathogens

Feces are extracted from the pig's rectum manually and placed in a sterile container. About a 1.5 cm diameter portion of the specimen was mixed thoroughly in 10 ml of 0.85% saline. The mixture is then strained slowly through a wire mesh strainer into a 15 ml conical centrifuge tube and centrifuged at 650 x g for 2 minutes to sediment the remaining fecal material. The supernatant is decanted carefully so as not to dislodge the sediment. and 10% buffered formalin was added to the 9 ml mark, followed by thorough mixing. The mixture is allowed to stand for 5 minutes. 4 ml of ethyl acetate is added to the mixture and the mixture is capped and mixed vigorously in an inverted position for 30 seconds. The cap is then removed to allow for ventilation and then replaced. The mixture is centrifuged at 500 x g for 1 minute (four layers should result: ethyl acetate, debris plug, formalin and sediment). The debris plug is rimmed using an applicator stick. The top three layers are carefully discarded by pouring them off into a solvent container. The debris attached to the sides of the tube is removed using a cotton applicator swab. The sediment is mixed in either a drop of formalin or the small amount of formalin which remains in the tube after decanting. Two separate drops are placed on a slide to which a drop of Lugol's iodine is added. Both drops are coverslipped and carefully examined for signs of pathogens, e.g., protozoan cysts of trophozoites, helminth eggs and larvae. Protozoan cyst identification is confirmed, when required, by trichrome staining.

B. Co-cultivation assay for detecting the presence of human and animal viruses in pig cells

Materials:

Cell lines

African green monkey kidney, (VERO), cell line American Type Culture Collection, (ATCC CCL81), human embryonic lung fibroblasts, (MRC-5) cell line American Type Culture Collection, (ATCC CCL 171), porcine kidney, (PK-15), cell line American Type Culture Collection, (ATCC CRL 33), porcine fetal testis, (ST), cell line American Type Culture Collection, (ATCC CRL 1746).

Medium, Antibiotics, and Other Cells, and Equipment

Fetal calf serum, DMEM, Penicillin 10,000 units/ml, Streptomycin 10 mg/ml, Gentamicin 50 mg/ml, guinea pig erythrocytes, chicken erythrocytes, porcine erythrocytes, Negative Control (sterile cell culture medium), Positive Controls: VERO and MRC-5 Cells: Poliovirus type 1 attenuated, (ATCC VR-1 92) and Measles virus, Edmonston strain, (ATCC VR-24), PK-1 5 and ST Cells: Swine influenza type A, (ATCC VR-99), Porcine Parvovirus, (ATCC VR-742), and Transmissible gastroenteritis of swine, (ATCC VR-743). Equipment: tissue Culture Incubator, Inverted Microscope, Biological Safety Cabinet.

These materials can be used in a co-cultivation assay (a process whereby a test article is inoculated into cell lines (VERO, MRC-5, PK1 5, and ST) capable of detecting a broad range of human, porcine and other animal viruses). Hsuing, G.D., "Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals" *in* Diagnostic Virology, 1982 (Yale University Press, New Haven, CT, 1982).

Experimental Design and Methodology:

A total of three flasks (T25) of each cell line are inoculated with at least 1 ml of test article. Three flasks of each cell line can also be inoculated with the appropriate sterile cell culture medium as a negative control. Positive control viruses are inoculated into three flasks of each cell line. After an absorption period, the inoculate is removed and all flasks incubated at 35-37°C for 21 days. All flasks are observed at least three times per week for the development of cytopathic effects, (CPE), of viral origin. Harvests are made from any flasks inoculated with the test article that show viral CPE.

At Day 7 an aliquot of supernatant and cells from the flasks of each test article are collected and at least 1 ml is inoculated into each of three new flasks of each cell line. These subcultures are incubated at 35-37°C for at least 14 days. All flasks are observed and tested as described above.

At Day 7, the flasks from each test article are also tested for viral hemadsorption, (HAd), using guinea pig, monkey and chicken erythrocytes at 2-8°C and 35-37°C at 14 days postinoculation.

At Day 21, if no CPE is noted, an aliquot of supernatant from each flask is collected, pooled, and tested for viral hemagglutination, (HA), using guinea pig, monkey, and chicken erythrocytes at 2-8°C and 35-37°C. Viral identification is based on characteristic viral cytopathic effects (CPE) and reactivity in HA testing.

The test samples are observed for viral cytopathic effects in the following manner: All cultures are observed for viral CPE at least three times each week for a minimum of 21 days incubation. Cultures are removed from the incubator and observed using an inverted microscope using at least 40X magnification. 100X or 200X magnification is used as appropriate. If any abnormalities in the cell monolayers, including viral CPE, are noted or any test articles cause total destruction of the cell monolayer, supernatant and cells are collected from the flasks and samples are subcultured in additional flasks of the same cell line. Samples can be stored at -60° to -80°C until subcultured. After 7 and 14 days incubation, two blind passages are made of each test article by collecting supernatant and cells from all flasks inoculated with each sample. Samples can be stored at -60° to -80°C until subcultured.

Hemadsorbing viruses are detected by the following procedure: after 21 days of incubation, a hemadsorption test is performed on the cells to detect the presence of hemadsorbing viruses. The cells are washed 1-2 times with approximately 5 mls of PBS. One to two mls of the appropriate erythrocyte suspension (either guinea pig, porcine, or chicken erythrocytes), prepared as described below, is then added to each flask. The flasks are then incubated at 2-8°C for 15-20 minutes, after which time the unabsorbed erythrocytes are removed by shaking the flasks. The erythrocytes are observed by placing the flasks on the lowered stage of a lab microscope and viewing them under low power magnification. A negative result is indicated by a lack of erythrocytes adhering to the cell monolayer. A positive result is indicated by the adsorption of the erythrocytes to the cell monolayer.

Hemagglutination testing, described in detail below, is also performed after 21 days of incubation of the subcultures. Viral isolates are identified based on the cell line where growth was noted, the characteristics of the viral CPE, the hemadsorption reaction, and hemagglutination reactions, as appropriate. The test article is considered negative for the presence of a viral agent, if any of the cell lines used in the study demonstrate viral, CPE, HA, or HAd in a valid assay.

C. Procedure for preparing and maintaining cell lines used to detect viruses in pig cells

Materials:

Fetal calf serum (FCS), DMEM, Penicillin 10,000 unit/ml, Streptomycin 10 mg/ml, Gentamicin 50 mg/ml, T25 tissue culture flasks, tissue culture incubator (5% CO_2, 37°C)

Procedure:

Aseptic techniques are followed when performing inoculations and transfers. All inoculations and transfers are performed in a biological safety cabinet. Media is prepared by adding 10% FCS for initial seeding, 5% FCS for maintenance of cultures, as well as 5.0 ml of penicillin/streptomycin and 0.5 ml of gentamicin per 500 ml media. Sufficient media is added to cover the bottom of a T25 tissue culture flask. The flask is seeded with the desired cell line and incubated at 37°C, 5% CO_2 until cells are 80 to 100% confluent. The flasks are then inoculated with virus (QCP25).

D. Preparation of erythrocyte (rbc) suspensions used in hemadsorption (HAd) and hemagglutination (HA) virus detection testing

Materials:

Phosphate buffered saline, (PBS), pH 7.2, guinea pig erythrocytes stock solution, porcine erythrocytes stock solution, chicken erythrocytes stock solution, sterile, disposable centrifuge tubes, 15 or 50 ml Laboratory centrifuge

Procedure:

An appropriate amount of erythrocytes (rbc) is obtained from stock solution. The erythrocytes are washed 3 times with PBS by centrifugation at approximately 1000 x g for 10 minutes. A 10% suspension is prepared by adding 9 parts of PBS to each one part of packed erythrocytes. The 10% rcb suspensions are stored at 2-8°C for no more than one week. 0.5% ecb suspensions are prepared by adding 19 parts of PBS to each one part of 10% rbc suspension. Fresh 0 5% rbc suspensions are prepared prior to each day's testing.

Hemagglutination (HA) test

A hemagglutination test is a test that detects viruses with the property to agglutinate erythrocytes, such as swine influenza type A, parainfluenza, and encephalomyocarditus viruses, in the test article. Hsuing, G.D. (1982) Diagnostic Virology (Yale University Press, New Haven, CT);. Stites, Daniel P and Terr, Abba I, (1991), Basic and Clinical Immunology (Appleton & Lange, East Norwalk, CT).

Materials:

Supernatants from flasks of the VERO cell line, MRC-5 inoculated with the test article, flasks of positive and negative controls, phosphate buffered saline (PBS), pH 7.2, guinea pig erythrocytes (GPRBC), 0.5% suspension in PBS, chicken erythrocytes (CRBC), 0.5% suspension in PBS, porcine erythrocytes (MRBC), 0.5% suspension in PBS

Procedure:

All sample collection and testing is performed in an approved biological safety cabinet. 0.5% suspensions of each type of erythrocytes are prepared as described above. The HA test on all cell lines inoculated with samples of the test articles at least 14 days post-inoculation. Positive and negative control cultures are included for each sample and monolayers are examined to ensure that they are intact prior to collecting samples.

At least 1 ml of culture fluid from each flask inoculated with the test article is collected and pooled. 1 ml samples from the negative and positive control cultures are also collected and pooled. A set of tubes is labeled with the sample number and type of erythrocyte (distinguish positive and negative suspension) to be added. Racks may be labeled to differentiate the type of erythrocyte. 0.1 ml of sample is added to each tube. 0.1 ml of the appropriate erythrocyte suspension is added to each tube. Each tube is covered with parafilm and mixed thoroughly. One set of tubes is incubated at 2-8°C until tight buttons form in the negative control in about 30-60 minutes. Another set of tubes is incubated at 35-37°C until tight buttons form in the negative control in about 30-60 minutes.

Formation of a tight button of erythrocytes indicates a negative result. A coating of the bottom of the tube with the erythrocytes indicates a positive result.

E. Methods used for fluorescent antibody stain of cell suspensions obtained from flasks used in detection of viruses in porcine cells using cell culture techniques (as described in Sections B and C)

Materials:

Pseudorabies, parvovirus, enterovirus. adenovirus, transmissible Gastroenteritis Virus. bovine viral diarrhea, encephalomyocarditus virus, parainfluenza, vesicular stomatitis virus., microscope slides, PBS, incubator with humidifying chamber at 36°C, Evan's blue coutner stain, DI Water, fluorescent microscope, trypsin, serum containing media, acetone, T25 Flask.

Procedure:

Cells (described in Sections B and C) are trypsinized to detach them from the T25 flask and sufficient media is added to neutralize trypsin activity. A drop of cell suspension is placed on each microscope slide and allowed to air dry. A slide for each fluorescent antibody

is prepared. Cells are fixed by immersion in acetone for five minutes. Each fluorescent antibody solution is placed on each slide to cover cells and the slides are incubated in humidifying chamber in incubator at 36°C for 30 minutes. The slides are then washed in PBS for five minutes. The wash is repeated in fresh PBS for five minutes followed by a rinse with DI water.

The cells are counterstained by placing Evan's blue solution on each slide to cover cells for five minutes at room temperature. The slides are then washed in PBS for five minutes. The wash is repeated in fresh PBS for five minutes followed by a rinse with DI water. The slides are then allowed to air dry. Each slide is inspected under a fluorescent microscope. Any fluorescent inclusion bodies characteristic of infection are considered a positive result for the presence of virus.

F. *Procedures for Defining Bacteremic Pigs*

Materials:

Anaerobic BMB agar (5% sheep blood, vitamin K and hemin [BMB/blood]), chocolate Agar with Iso Vitalex, Sabaroud dextrose agar/Emmons, 70% isopropyl alcohol swabs, betadine solution, 5% CO_2 incubator at 35-37°C, anaerobic blood agar plate, gram stain reagents (Columbia Broth Media), aerobic blood culture media (anaerobic brain heart infusion with vitamin K& hemin), septicheck media system, vitek bacterial identification system, laminar flow hood, microscope, and bacteroids and Bacillus stocks

Procedure:

Under a laminar flow hood, disinfect the tops of bottles for aerobic and anaerobic blood cultures of blood obtained from pig with 70% isopropyl alcohol, then with betadine The rubber stopper and cap from the aerobic blood culture bottle are removed and a renal septicheck media system is attached to the bottle. The bottles are incubated in 5% CO_2 for 21 days at 35-37°C, and observed daily for any signs of bacterial growth (i.e. gas bubbles, turbidity, discoloration or discrete clumps). Negative controls consisting of 5cc of sterile saline in each bottle and positive controls consisting of Bacillus subtilis in the aerobic bottle and Bacteriodes Vulgaris in the anaerobic bottle are used. If signs of bacterial growth are observed, a Gram stain is prepared and viewed microscopically at 100x oil immersion for the presence of any bacteria or fungi. The positive bottles are then subcultured onto both chocolate agar plates with Iso Vitlex and onto BMB plates. The chocolate plate is incubated at 35-37°C in 5% $C0_2$ for 24 hours and the BMB anaerobically at 35-37°C for 48 hours. Any yeast or fungi that is in evidence at gram stain is subcultured onto a Sabaroud dextrose/Emmons plate. The Vitek automated system is used to identify bacteria and yeast. Fungi are identified via their macroscopic and microscopic characteristic. If no signs of

growth are observed at the end of 21 days, gram stain is prepared and observed microscopically for the presence of bacteria and fungi.

Absence of growth in the negative control bottles and presence of growth in the positive control bottles indicates a valid test. The absence of any signs of growth in both the aerobic and anaerobic blood culture bottles, as well as no organisms seen on gram stain indicates a negative blood culture. The presence and identification of microorganism(s) in either the aerobic or anaerobic blood culture bottle indicates of a positive blood culture; this typically is due to a bacteremic state.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

1. A porcine hepatocyte, which, in unmodified form, has at least one antigen on the cell surface which is capable of stimulating an immune response against the cell in a xenogeneic subject, wherein the antigen on the cell surface is altered to inhibit rejection of the cell when introduced into the xenogeneic subject.

2. The porcine hepatocyte of claim 1, wherein the antigen on the cell surface which is altered is an MHC class I antigen.

3. The porcine hepatocyte of claim 2, which is contacted prior to introduction into a xenogeneic subject with at least one anti-MHC class I antibody, or fragment or derivative thereof, which binds to the MHC class I antigen on the cell surface but does not activate complement or induce lysis of the cell.

4. The porcine hepatocyte of claim 3, wherein the anti-MHC class I antibody is an anti-MHC class I $F(ab')_2$ fragment.

5. The porcine hepatocyte of claim 4, wherein the anti-MHC class I $F(ab')_2$ fragment is a $F(ab')_2$ fragment of a monoclonal antibody PT85.

6. The porcine hepatocyte of claim 1, wherein the xenogeneic subject is a human.

7. The porcine hepatocyte of claim 1, which is obtained from an embryonic pig.

8. A porcine hepatocyte having an antibody, antibody fragment, or derivative, bound to its surface.

9. The porcine hepatocyte of claim 8, wherein the antibody is an anti-MHC class I antibody.

10. The porcine hepatocyte of claim 9 wherein the anti-MHC class I antibody is an anti-MHC class I $F(ab')_2$ fragment.

11. The porcine hepatocyte of claim 10, wherein the anti-MHC class I $F(ab')_2$ fragment is a $F(ab')_2$ fragment of a monoclonal antibody PT85.

12. The porcine hepatocyte of claim 8, which is obtained from an embryonic pig.

13. An isolated hepatocyte obtained from a pig which is essentially free from organisms or substances which are capable of transmitting infection or disease to a xenogeneic recipient of the cells.

14. The hepatocyte of claim 13, which is isolated from a pig which is essentially free from at least one organism selected from the group consisting of parasites, bacteria, mycoplasma, and viruses.

15. The hepatocyte of claim 13, which is obtained from an embryonic pig.

16. The hepatocyte of claim 13, wherein the hepatocyte has an antibody, antibody fragment, or derivative, bound to its surface.

17. An isolated population of porcine hepatocytes which, in unmodified form, have at least one antigen on the surface of the cells which is capable of stimulating an immune response against the cells in a xenogeneic subject, wherein the antigen on the surface of the cells is altered to inhibit rejection of the cells when introduced into the xenogeneic subject.

18. The isolated population of porcine hepatocytes of claim 17, which is obtained from an embryonic pig.

19. The isolated population of porcine hepatocytes of claim 17, wherein the antigen on the cell surface which is altered is an MHC class I antigen.

20. The isolated population of porcine hepatocytes claim 19, which is contacted prior to transplantation into a xenogeneic subject with at least one anti-MHC class I antibody, or fragment or derivative thereof, which binds to the MHC class I antigen on the cell surface but does not activate complement or induce lysis of the cell.

21. The isolated population of porcine hepatocytes of claim 17, which is maintained in culture.

22. A composition comprising a population of porcine hepatocytes and antibodies, antibody fragments, or derivatives, which bind an antigen on the surface of the porcine hepatocytes.

23. The composition of claim 22, which is contained within a delivery device.

24. The composition of claim 23, wherein the delivery device is a syringe.

25. An isolated population of porcine hepatocytes which is obtained from a pig which is essentially free from organisms or substances which are capable of transmitting infection or disease to a xenogeneic recipient of the cells.

26. The isolated population of porcine hepatocytes of claim 25, which is isolated from a pig which is essentially free from at least one organism selected from the group consisting of parasites, bacteria, mycoplasma, and viruses.

27. The isolated population of porcine hepatocytes of claim 25, which is obtained from an embryonic pig.

28. The isolated population of porcine hepatocytes of claim 25, wherein the hepatocytes have antibodies, antibody fragments, or derivatives, bound to their surfaces.

29. A composition comprising porcine hepatocytes obtained from a pig which is essentially free from organisms or substances which are capable of transmitting infection or disease to a xenogeneic recipient of the cells and antibodies, antibody fragments, or derivatives, which bind an antigen on the surface of the porcine hepatocytes.

30. The composition of claim 29, which is contained within a delivery device.

31. The composition of claim 30, wherein the delivery device is a syringe.

32. A method for treating a disorder characterized by insufficient liver function in a subject, comprising administering to a subject having the disorder an isolated population of porcine hepatocytes which, in unmodified form, have at least one antigen on the surface of the cells which is capable of stimulating an immune response against the cells in a xenogeneic subject, wherein the antigen on the surface of the cells is altered to inhibit rejection of the cells when introduced into the xenogeneic subject.

33. The method of claim 32, wherein the porcine hepatocytes are obtained from an embryonic pig.

34. The method of claim 32, wherein the subject is a human.

35. The method of claim 32, wherein the disorder is familial hypercholesterolemia.

36. The method of claim 32, wherein the disorder is a genetic disease involving a liver enzyme.

37. The method of claim 3, wherein the genetic disease is Crigler-Najjar Syndrome Type I or ornithine transcarbamoylase deficiency.

38. The method of claim 32, wherein the disorder is a genetic disease involving a protein secreted by the liver.

39. The method of claim 38, wherein the genetic disease is hereditary emphysema or hemophilia.

40. The method of claim 32, wherein the disorder is acute liver failure.

41. The method of claim 32, wherein the disorder is chronic liver failure.

42. The method of claim 32, wherein the disorder is hepatitis.

43. The method of claim 32, wherein the porcine hepatocytes are contacted prior to introduction into the subject with at least one molecule which binds to at least one antigen on the cell surface which is capable of stimulating an immune response against the cells in the subject to alter the antigen on the cell surface to inhibit rejection of the cells when introduced into the subject.

44. The method of claim 43, wherein the antigen on the surface of the porcine hepatocytes which is altered is an MHC class I antigen.

45. The method of claim 44, wherein the porcine hepatocytes are contacted prior to introduction into a subject with at least one anti-MHC class I antibody, or fragment or derivative thereof, which binds to the MHC class I antigen on the cell surface but does not activate complement or induce lysis of the cells.

46. The method of claim 45, wherein the anti-MHC class I antibody is an anti-MHC class I $F(ab')_2$ fragment.

47. The method of claim 46, wherein the anti-MHC class I $F(ab')_2$ fragment is a $F(ab')_2$ fragment of a monoclonal antibody PT85.

48. The method of claim 32, further comprising administering an immunosuppressive agent to the subject.

49. A method for treating a disorder characterized by insufficient liver function in a subject, comprising administering to a subject having the disorder an isolated population of porcine hepatocytes obtained from a pig which is essentially free from organisms or substances which are capable of transmitting infection or disease to a xenogeneic recipient of the cells.

50. The method of claim 49, wherein the porcine hepatocytes are obtained from a pig which is essentially free from at least one organism selected from the group consisting of parasites, bacteria, mycoplasma, and viruses.

51. The method of claim 49, wherein the porcine hepatocytes are obtained from an embryonic pig.

52. The method of claim 49, wherein the subject is a human.

53. The method of claim 49, wherein the disorder is familial hypercholesterolemia.

54. The method of claim 49, wherein the disorder is a genetic disease involving a liver enzyme.

55. The method of claim 54, wherein the genetic disease is Crigler-Najjar Syndrome Type I or ornithine transcarbamoylase deficiency.

56. The method of claim 49, wherein the disorder is a genetic disease involving a protein secreted by the liver.

57. The method of claim 56, wherein the genetic disease is hereditary emphysema or hemophilia.

58. The method of claim 49, wherein the disorder is acute liver failure.

59. The method of claim 49, wherein the disorder is chronic liver failure.

60. The method of claim 49, wherein the disorder is hepatitis.

61. The method of claim 49, further comprising administering an immunosuppressive agent to the subject.

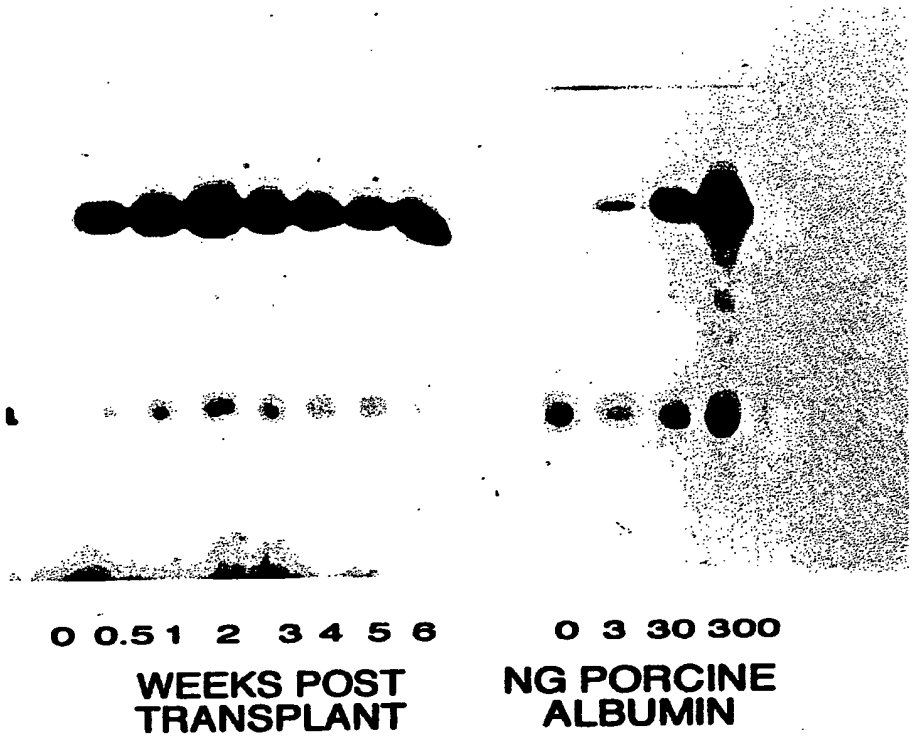

0 0.5 1 2 3 4 5 6
WEEKS POST TRANSPLANT
0 3 30 300
NG PORCINE ALBUMIN

FIG. 1

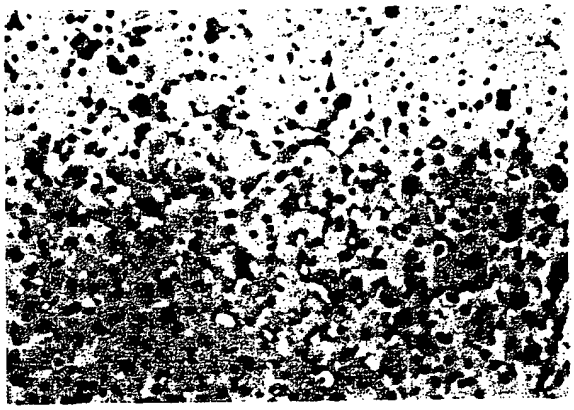


FIG.2A

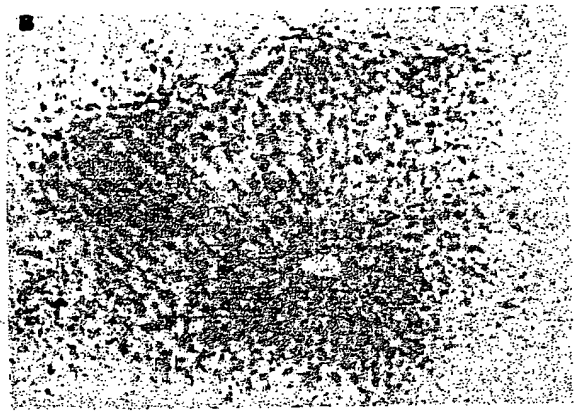


FIG.2B

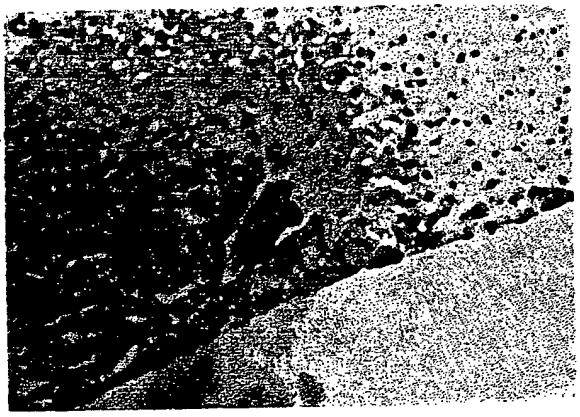


FIG.2C

FIG.2D

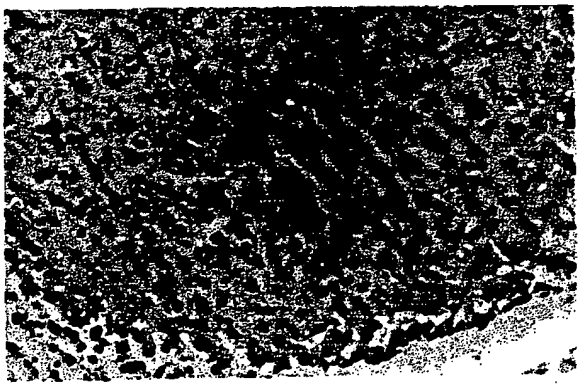


FIG.2E

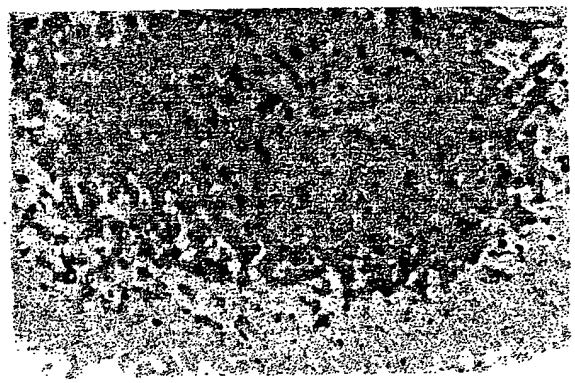


FIG.2F

FIG. 3

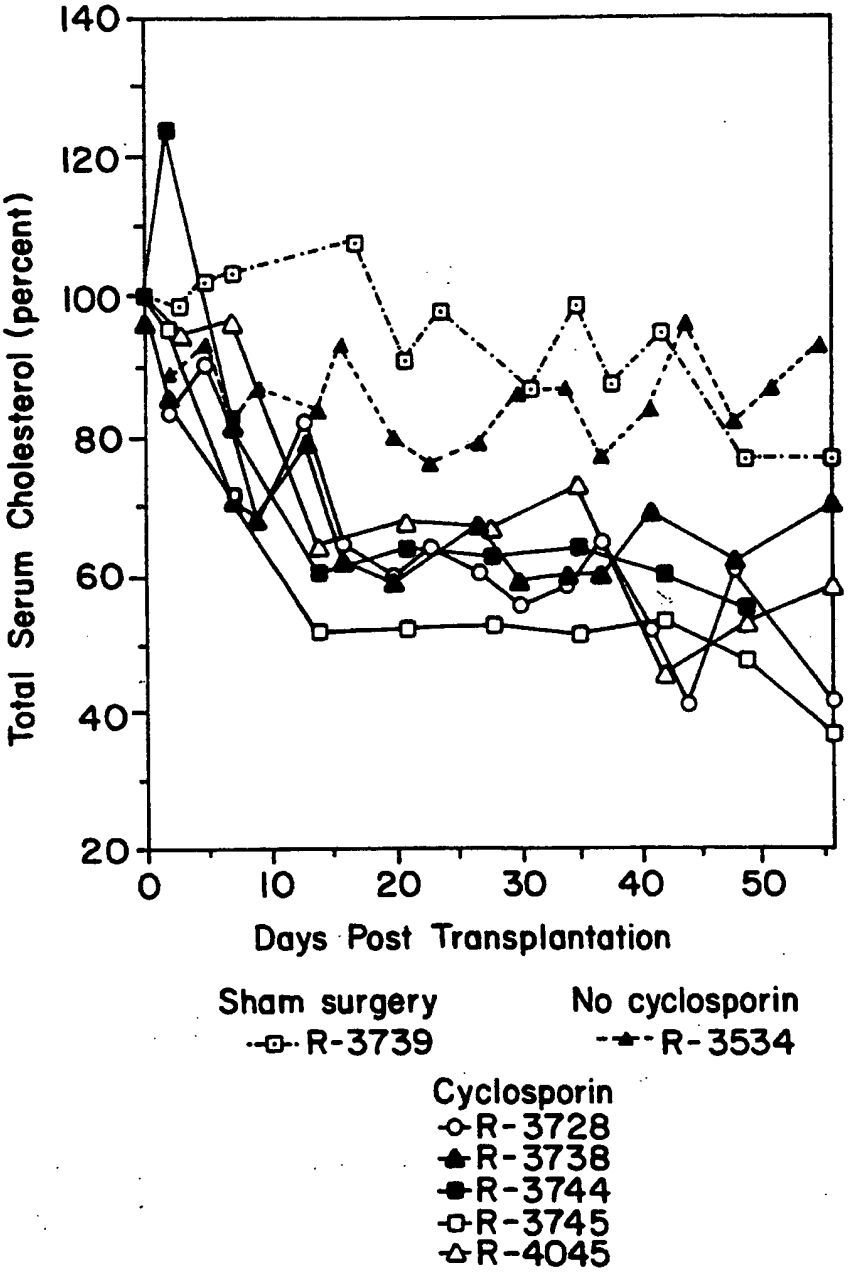

140
120
100
80
60
40
20
Total Serum Cholesterol (percent)
0
10
20
30
40
50
Days Post Transplantation
Sham surgery
R-3739
No cyclosporin
R-3534
Cyclosporin
R-3728
R-3738
R-3744
R-3745
R-4045

FIG. 4

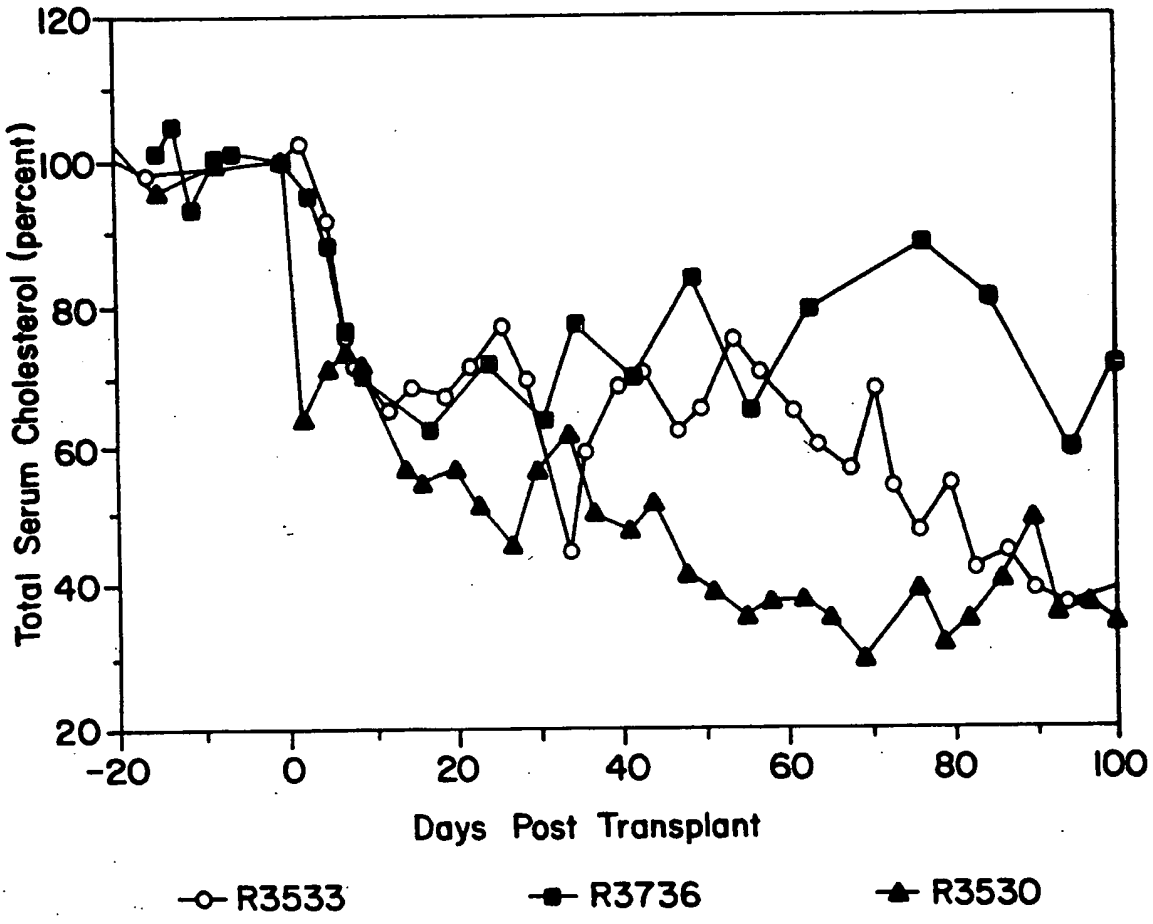

120
100
80
60
40
20
Total Serum Cholesterol (percent)
-20
0
20
40
60
80
100
Days Post Transplant
R3533
R3736
R3530

FIG. 5

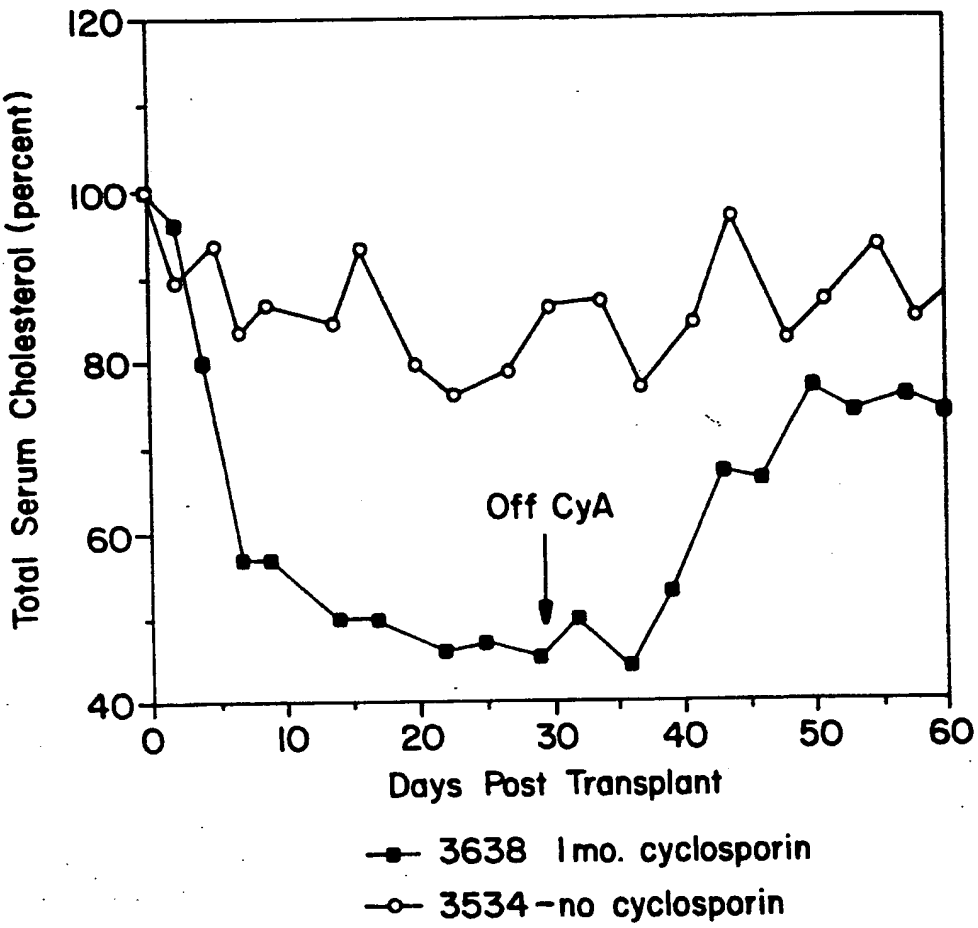

120
100
80
60
40
Total Serum Cholesterol (percent)
0
10
20
30
40
50
60
Days Post Transplant
Off CyA
3638 1mo. cyclosporin
3534-no cyclosporin

FIG. 6

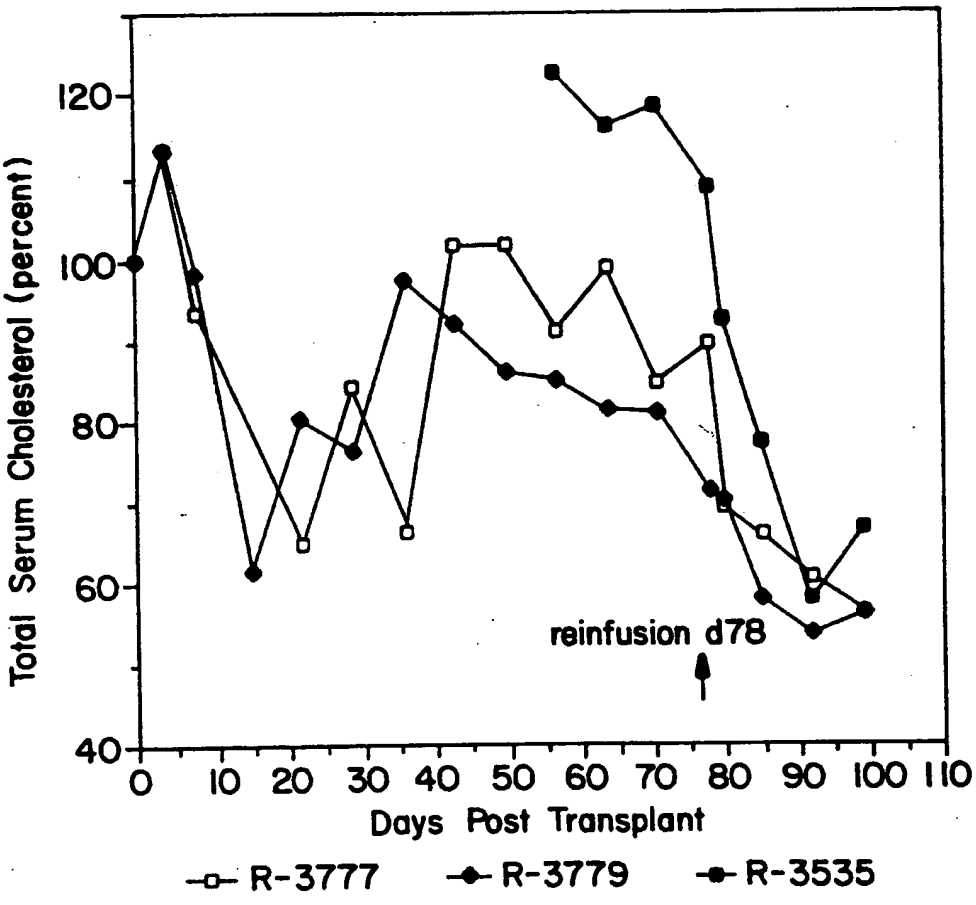

Total Serum Cholesterol (percent)
120
100
80
60
40
0
10
20
30
40
50
60
70
80
90
100
110
Days Post Transplant
reinfusion d78
R-3777
R-3779
R-3535

FIG. 7

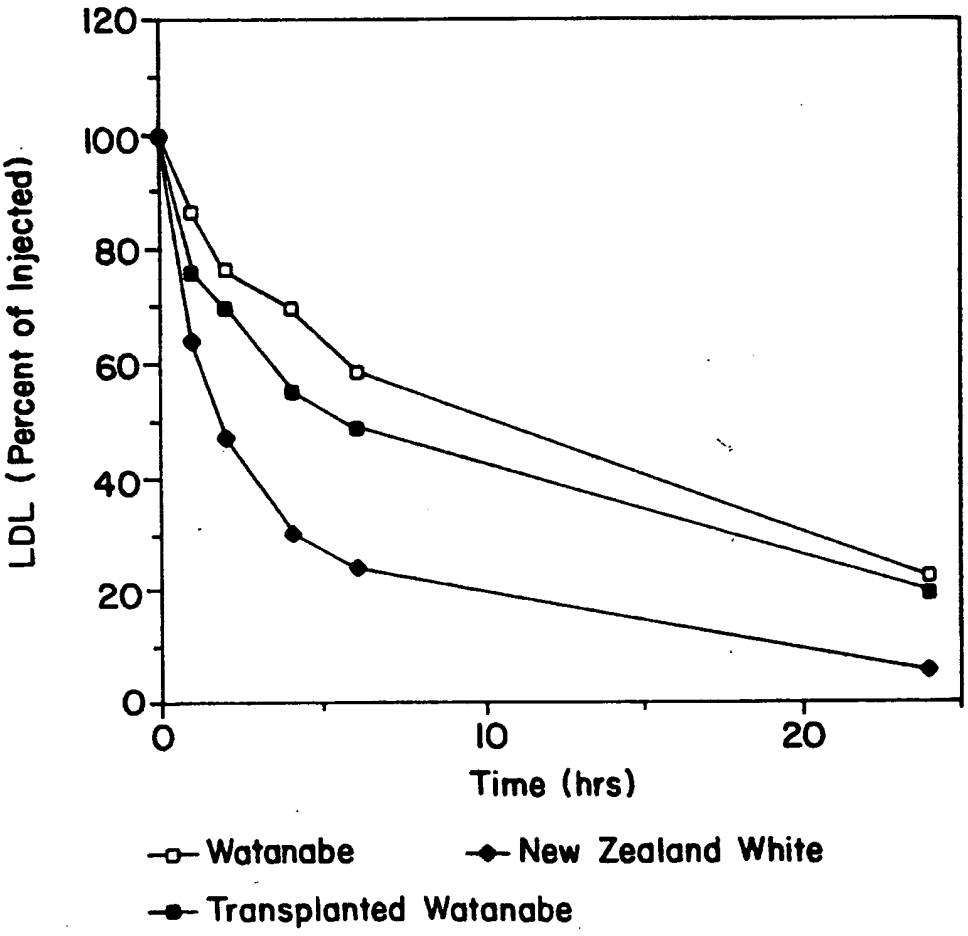

120
100
80
60
40
20
0
LDL (Percent of Injected)
0
10
20
Time (hrs)
Watanabe
New Zealand White
Transplanted Watanabe

FIG. 8

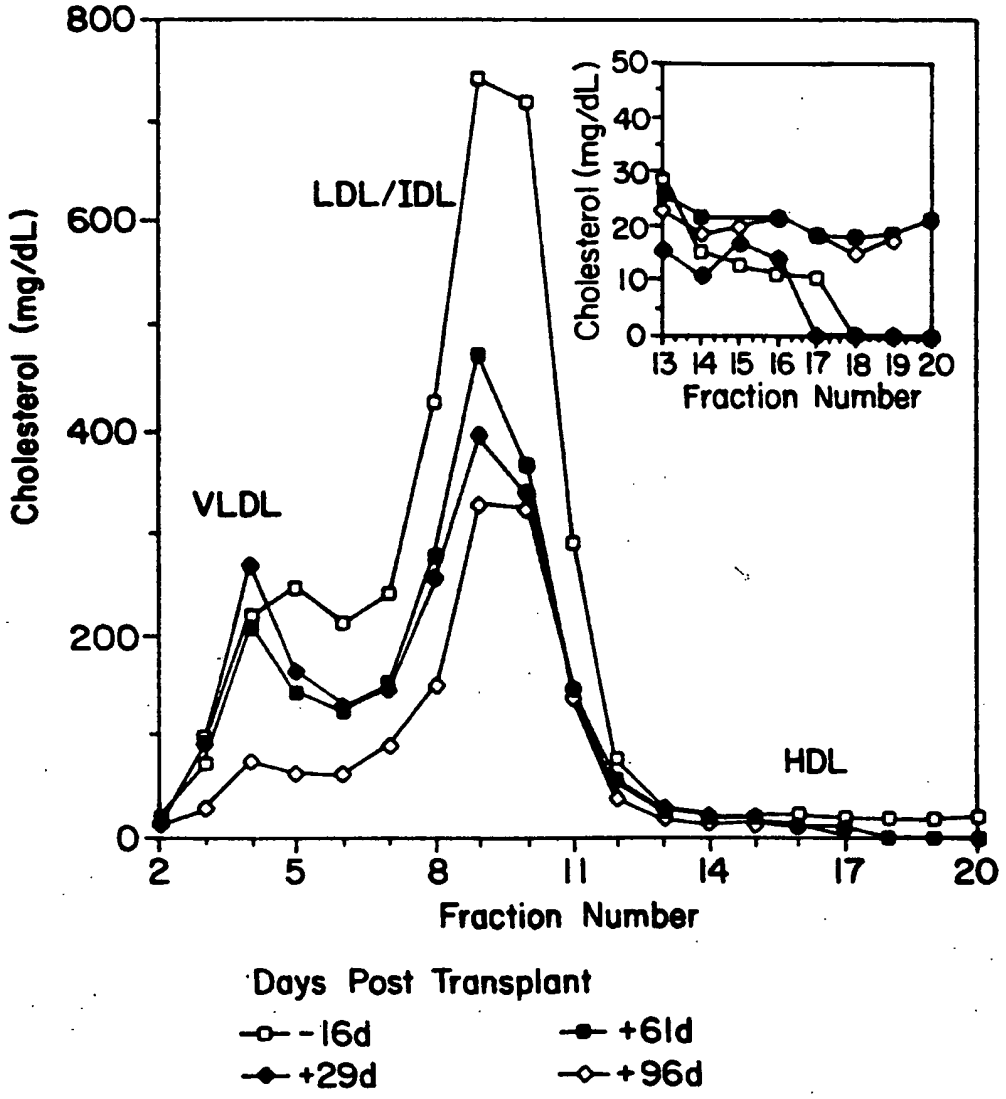

800
600
400
200
0
Cholesterol (mg/dL)
LDL/IDL
VLDL
HDL
2
5
8
11
14
17
20
Fraction Number
50
40
30
20
10
0
Cholesterol (mg/dL)
13 14 15 16 17 18 19 20
Fraction Number
Days Post Transplant
-16d
+29d
+61d
+96d

FIG. 9

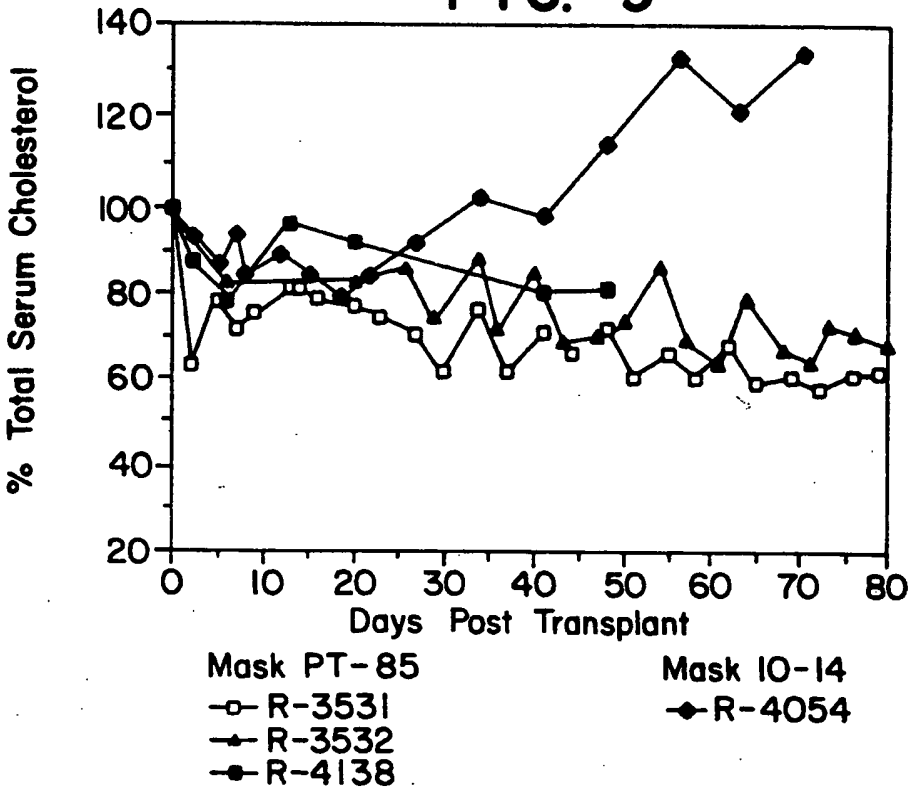

% Total Serum Cholesterol
140
120
100
80
60
40
20
0
10
20
30
40
50
60
70
80
Days Post Transplant
Mask PT-85
R-3531
R-3532
R-4138
Mask 10-14
R-4054

FIG. 10

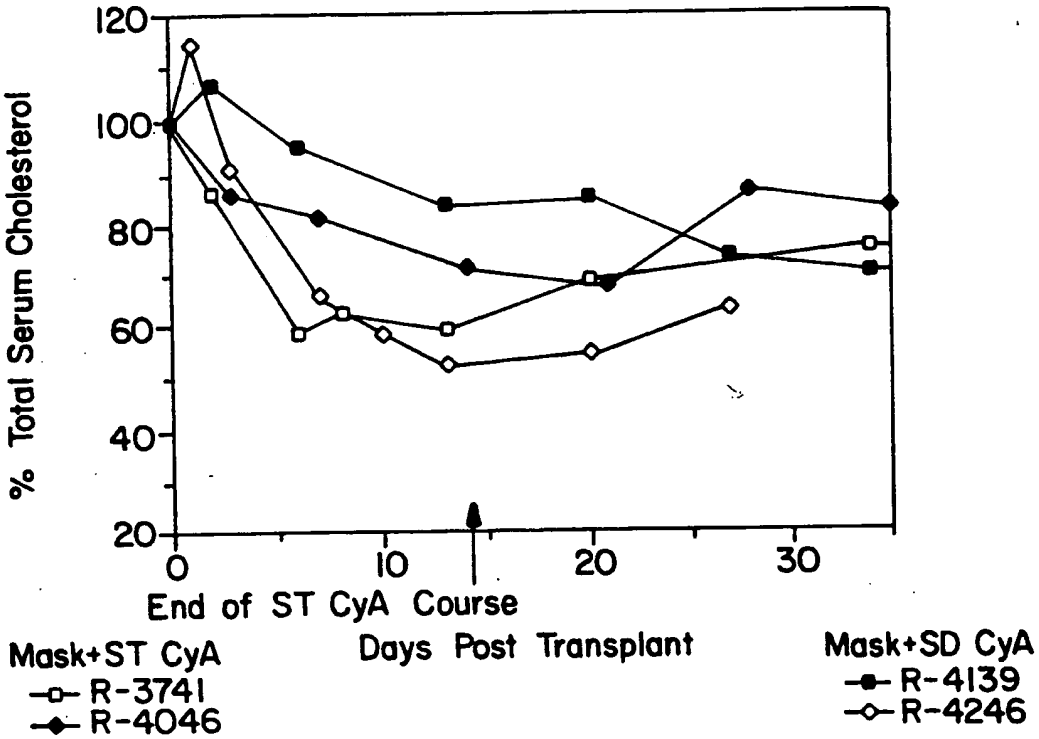

% Total Serum Cholesterol
120
100
80
60
40
20
0
10
20
30
End of ST CyA Course
Days Post Transplant
Mask+ST CyA
R-3741
R-4046
Mask+SD CyA
R-4139
R-4246

FIG. 11

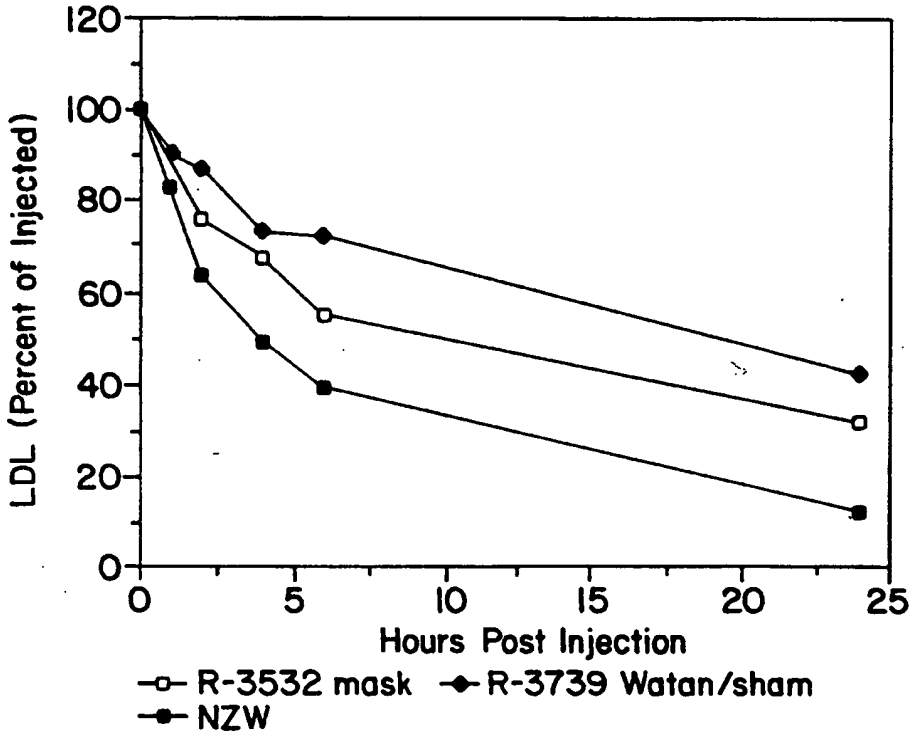
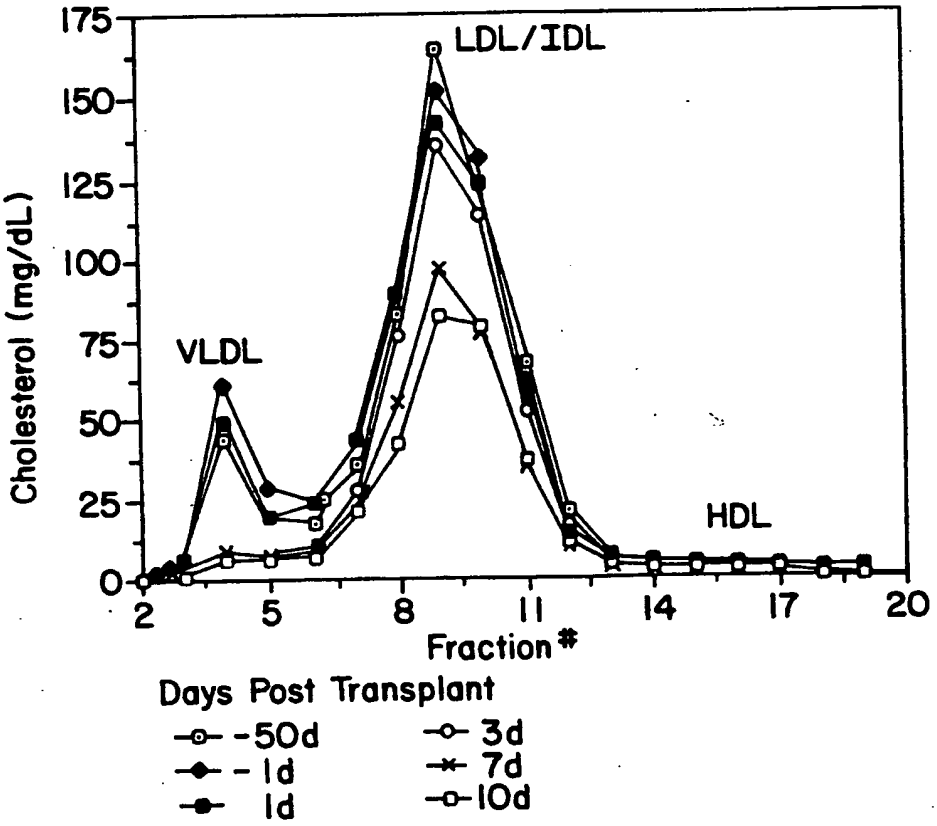

120
100
80
60
40
20
0
LDL (Percent of Injected)
0
5
10
15
20
25
Hours Post Injection
R-3532 mask
R-3739 Watan/sham
NZW

FIG. 12


175
150
125
100
75
50
25
0
Cholesterol (mg/dL)
LDL/IDL
VLDL
HDL
2
5
8
11
14
17
20
Fraction #
Days Post Transplant
-50d
-1d
1d
3d
7d
10d

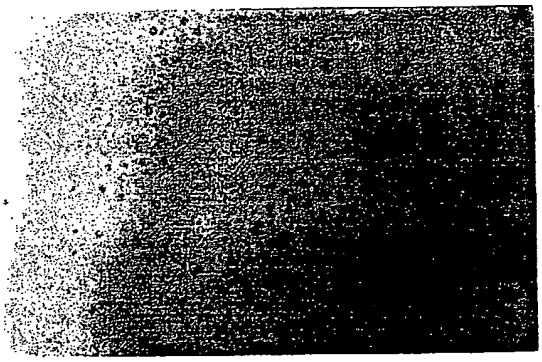


FIG.13A

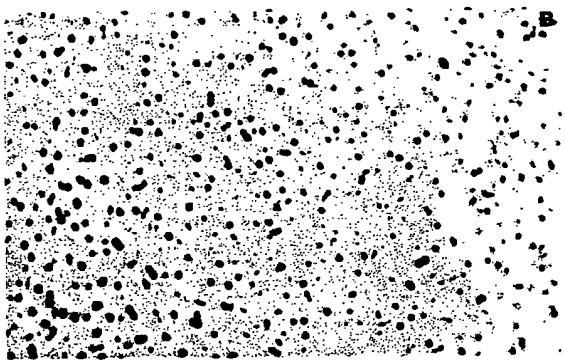


FIG.13B

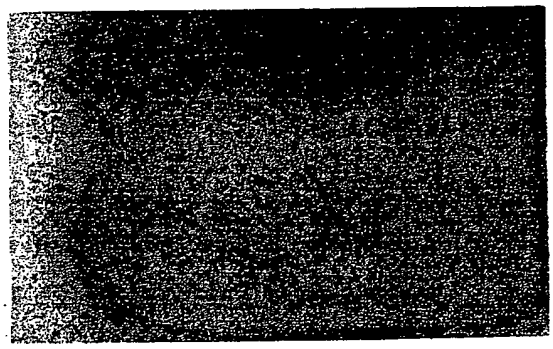


FIG.13C

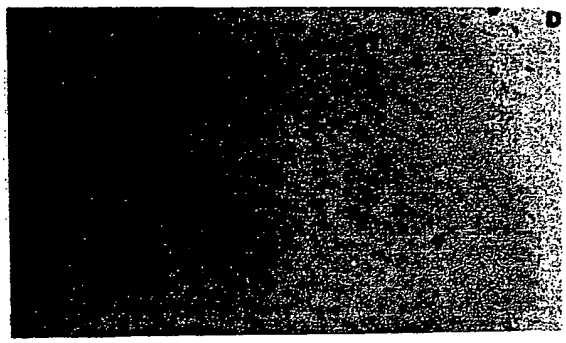


FIG.13D

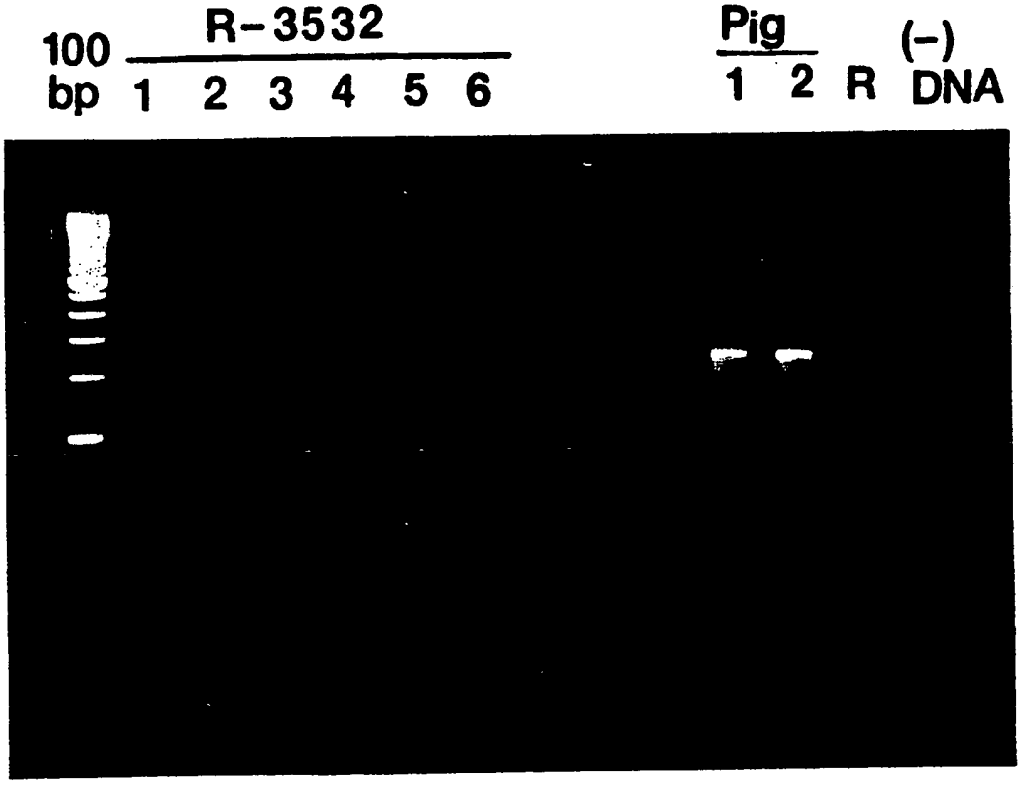


FIG.14

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 96/07590

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/06 A61K35/407

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 02188 (OKLAHOMA MEDICAL RESEARCH FOUNDATION & YALE UNIVERSITY) 4 February 1993 see page 25, line 22 - page 30, line 18 see claims ---	1,2,6, 17,19, 21,32,34
Y	WO,A,92 04033 (THE GENERAL HOSPITAL CORPORATION) 19 March 1992 see example 3 see claims --- -/--	1-12, 17-24, 32-47

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 August 1996	03.09.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Nooij, F

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CELL TRANSPLANTATION, vol. 3, no. 4, July 1994, NEW YORK, NY, USA, pages 283-290, XP000578335 M. HAGIHARA ET AL.: "Effects of iso and xeno fetal liver fragments transplantation on acute and chronic liver failure in rats." see abstract	1-12, 17-24, 32-47
A	SCIENCE, vol. 252, no. 5013, 21 June 1991, WASHINGTON, DC, USA, pages 1700-1702, XP002011037 D. FAUSTMAN ET AL.: "Prevention of xenograft rejection by masking donor HLA class I antigens." see the whole document	1-61
A	SCIENCE, vol. 254, no. 5039, 20 December 1991, WASHINGTON, DC, USA, pages 1802-1805, XP002011038 J. CHOWDHURY ET AL.: "Long-term improvement of hypercholesterolemia after ex in vivo gene therapy in LDLR-deficient rabbits." see abstract	1-61
A	WO,A,94 27622 (THE JOHNS HOPKINS UNIVERSITY) 8 December 1994 see figure see claims	1-61
A	TRANSPLANTATION, vol. 55, no. 4, April 1993, BALTIMORE, MD, USA, pages 940-946, XP000578337 X. LI ET AL.: "Use of donor beta2-microglobulin-deficient transgenic mouse liver cells for isografts, allografts, and xenografts." see abstract	1-61
P,X	WO,A,95 26740 (DIACRIN, INC.) 12 October 1995 see example II see claims	1-12, 17-24, 32-48
1 P,X	WO,A,95 33828 (DIACRIN, INC.) 14 December 1995 see examples	1,6,7, 17-19, 21,32-34

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/07590

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 32 - 61 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/US 96/07590

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9302188	04-02-93	CA-A-	2113089	04-02-93
		EP-A-	0591462	13-04-94
		JP-T-	6506604	28-07-94
		AU-B-	4657993	24-01-94
		WO-A-	9400560	06-01-94
WO-A-9204033	19-03-92	US-A-	5283058	01-02-94
		AU-B-	656150	27-01-95
		AU-B-	8510491	30-03-92
		CA-A-	2090009	01-03-92
		EP-A-	0550482	14-07-93
		JP-T-	6503810	28-04-94
WO-A-9427622	08-12-94	AU-B-	7044594	20-12-94
		EP-A-	0700297	13-03-96
WO-A-9526740	12-10-95	AU-B-	2202195	23-10-95
WO-A-9533828	14-12-95	AU-B-	2587995	04-01-96